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Article

Electrical Activity Suppresses Axon Growth through $Ca_v1.2$ Channels in Adult Primary Sensory Neurons

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Summary

Background: Primary sensory neurons of the dorsal root ganglia (DRG) regenerate their spinal cord axon if the peripheral nerve axon has previously been cut. This conditioning lesion confers axon growth competence to the neurons. However, the signal that is sensed by the cell upon peripheral lesion to initiate the regenerative response remains elusive.
Results: We show here that loss of electrical activity following peripheral deafferentiation is an important signal to trigger axon regrowth. We first verified that firing in sensory fibers, as recorded from dorsal roots in vivo, declined after peripheral lesioning but was not altered after central lesioning. We found that electrical activity strongly inhibited axon outgrowth in cultured adult sensory neurons. The inhibitory effect depended on the L-type voltage-gated Ca^{2+} channel current and involved transcriptional changes. After a peripheral lesion, the L-type current was consistently diminished and the L-type pore-forming subunit, $Ca_v1.2$, was downregulated. Genetic ablation of $Ca_v1.2$ in the nervous system caused an increase in axon outgrowth from dissociated DRG neurons and enhanced peripheral nerve regeneration in vivo.
Conclusions: Our data indicate that cessation of electrical activity after peripheral lesion contributes to the regenerative response observed upon conditioning and might be necessary to promote regeneration after central nervous system injury.

Introduction

Injured neurons regenerate their axons poorly in the adult mammalian central nervous system (CNS). However, stimulating their intrinsic growth competence enables neurons to regrow and to overcome the inhibitory environment created by factors present in the CNS myelin [1, 2]. In primary sensory neurons of the dorsal root ganglia (DRG), cutting their peripheral axon, which is called conditioning, triggers growth competence through activation of a new genetic program [3]. Under these circumstances, not only does the peripheral axon regrow in the peripheral nervous system (PNS), but their central axon coursing in the dorsal column of the CNS also regenerates [4–6]. The nature of the signal that ascends from the peripheral lesion site to the cell body to implement this growth program is poorly understood.

It has been proposed that a peripheral lesion may reactivate the growth program either by exposing the tip of the injured axon to growth-promoting positive signals present in the reactive PNS tissue or by interrupting growth-inhibiting retrograde signaling elicited from the peripheral target (reviewed in [7, 8]). Neurotrophic factors and cytokines, including nerve growth factor, neurotrophin 3, glial derived neurotrophic factor, and interleukin 6, contribute as extracellular positive signals [9, 10]. No negative signals elicited from the periphery have been identified so far.

Electrical activity, the basis of signal transmission in neuronal networks, has long been recognized as an important factor for shaping the development of the nervous system [11, 12]. For instance, normal levels of spontaneous activity are critical for correct pathfinding of motorneurons' axons in vivo [13]. In cultured embryonic neurons, electrical stimulation can modulate axonal extension in a cell-type-dependent manner [14–16]. We therefore asked whether electrical activity can suppress axon growth in sensory neurons in the adult stage and hinder their regeneration.

Electrical activity triggers an increase in intracellular Ca^{2+} concentration, which, by activating different signaling cascades, plays a pivotal role in modulating axon extension and guidance [11, 17]. Voltage-gated Ca^{2+} channels (VGCCs) open in response to membrane depolarization, constituting a major route of Ca^{2+} entry into the cell. The different types of VGCCs show distinct kinetics, subcellular localizations, and functional properties. VGCCs have also been implicated in regulating axon growth during neuronal development [18–20].

Here we show that electrical activity suppresses the growth program in functional adult primary sensory neurons via a specific voltage-gated Ca^{2+} channel current, namely the L-type current. Our findings support the conclusion that electrical activity governs the growth competence of adult DRG neurons. We propose that electrical silencing is an important cue in eliciting the conditioning effect.

Results

The Firing Rate of DRG Neurons In Vivo Decreases upon Peripheral, but Not Central, Axotomy

Primary sensory neurons convey sensory information from their peripheral targets to the spinal cord and brain. These neurons have no dendrites, but the peripheral branch acts as the receiving process, and the central branch, which typically has numerous ascending and descending collaterals, acts as the transmitting process. Importantly, whereas axotomy in the peripheral branch deprives neurons of sensory-evoked

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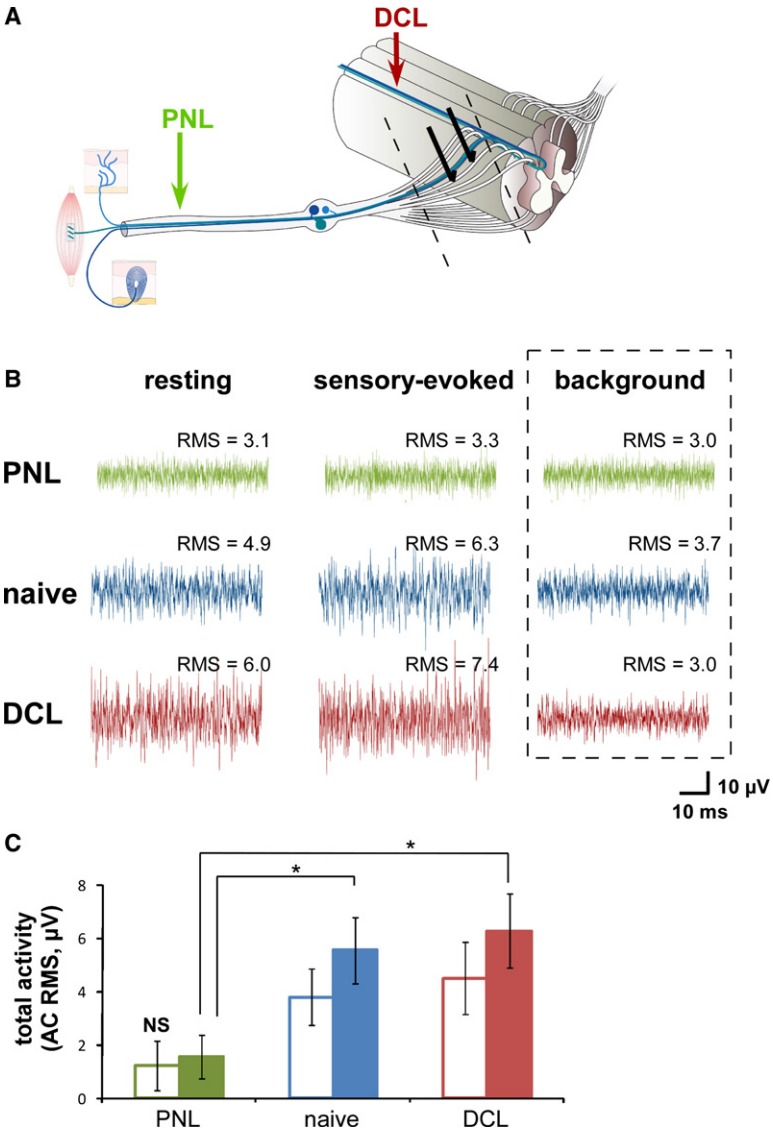


Figure 1. Axotomy in the Peripheral Branch, but Not in the Central Branch, Silences Dorsal Root Ganglia Neurons In Vivo

(A) Illustration of the experimental design. Hook electrodes were placed in the dorsal rootlets to record multifiber compound activity from sensory neurons. Sequential measurements were done in the intact dorsal rootlets and after combined distal and proximal ligations.

(B) Representative traces of each of the three experimental groups: naive (blue), peripheral nerve lesioned (PNL, green), and dorsal column lesioned (DCL, red) neurons. Recordings labeled "background" were obtained after proximal and distal ligations. The corresponding alternate current root-mean-square voltage values for each of the traces are indicated in the upper right corner.

(C) Data analysis comparing either resting activity levels (open bars) or sensory-evoked activity (filled bars) in each of the three experimental groups. The sensory responses are averaged from four conditions of continuous applied cutaneous (pinch, brush) or proprioceptive (bend, rotation) stimulation, described in the Experimental Procedures. Data points (mean ± standard error of the mean [SEM]; $n \geq 7$ dorsal rootlets from five rats for each condition) have had the intrinsic recording noise removed. One-way analysis of variance (ANOVA) on the three treatments (naive, DCL, PNL) was significant for both sensory and resting activity. Pairwise comparisons with Bonferroni corrections were significant for sensory but not resting activity. Resting activity: PNL-naive, not statistically different (NS), $p = 0.14$; PNL-DCL, NS, $p = 0.096$. Averaged sensory-evoked activity: PNL-naive, *$p = 0.016$; PNL-DCL, *$p = 0.018$. Individual sensory responses, not depicted in the figures, showed significant pairwise differences ($p < 0.05$) between the lesioned (PNL or DCL) and the naive in all cases except for joint rotation in the PNL group.

activity, axotomy in the central branch does not. Therefore, we predicted that DRG neurons fall silent after peripheral lesion but remain electrically active after central lesion.

In order to validate our assumption, we performed in vivo electrophysiological recordings of sensory neurons in rats subjected to either peripheral nerve lesion (PNL) or dorsal column lesion (DCL). The contralateral side of peripheral injured animals served as a control to determine normal levels of activity in naive DRG neurons. Two days after the rats' injury, using a pair of hook electrodes on the dorsal rootlets of L4 and L5 ganglia, we recorded action potentials in multiple sensory fibers (Figure 1A). Root-mean-square voltage was taken as a measure of spike activity. For each rootlet in each experimental condition, we examined resting activity (Figure 1B, left column) and the response to various sensory stimuli, namely touch, pinch, and static and dynamic joint bend (Figure 1B, middle column). To determine the contribution to overall firing rates of action potentials traveling both to and from the spinal cord, we ligated the dorsal rootlets distal and proximal to the electrode pair and repeated the recording. The background activity after combined distal and proximal ligation was considered to be the intrinsic noise level of the recording (Figure 1B, right column) and was subtracted from the calculated value for resting or sensory-evoked activity. We observed that resting ongoing activity was slightly reduced after PNL, but not after DCL (Figure 1C, unfilled bars), although differences did not reach significance in pairwise comparisons. However, in the case of sensory-evoked activity, which probably better resembles the activity levels experienced by awake and freely moving rats, the differences were more pronounced. We found that sensory-evoked activity was significantly suppressed after axotomy in the peripheral branch (Figure 1B, green traces compared with blue traces). By contrast, sensory-evoked activity was still present at normal levels after axotomy in the central branch (Figure 1B, red traces; Figure 1C, filled bars).

In conclusion, our findings are consistent with the idea that injury-induced changes in the firing of DRG neurons influence their ability to regenerate. After peripheral lesion, neurons fall silent and become growth competent, whereas after central lesion, neurons remain electrically active and growth incompetent.

Electrical Activity Inhibits Axon Outgrowth in Adult DRG Neurons

To investigate whether the observed differences in firing after PNL determine axon growth competence, we next assessed the effect of electrical activity on axon growth. We tested whether electrical activity affects neurite outgrowth in cultured adult sensory DRG neurons. Our studies focus on medium- and large-diameter (>30 μm) DRG neurons, the majority of

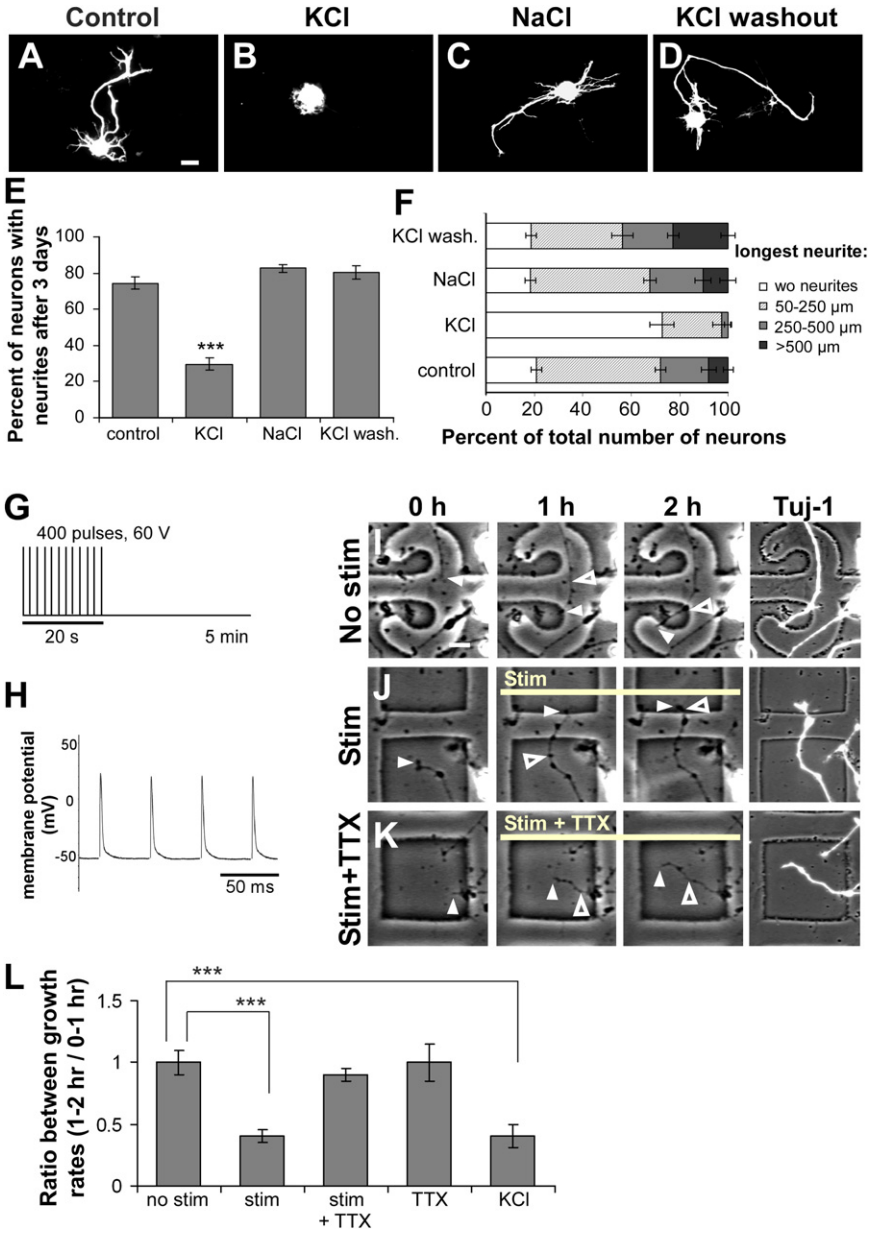


Figure 2. Neural Activity Inhibits Axon Outgrowth on Adult Dorsal Root Ganglion Neurons

(A–F) Neurons were isolated from adult rats and cultured on poly-lysine for 3 days in control (A), 40 mM KCl-containing (B) or 40 mM NaCl-containing (C) media.

(D) Neurons treated for 3 days with 40 mM KCl and subsequently replaced in control media for 3 additional days. Scale bar represents 25 μm.

(E) Quantification showing the percent of neurons bearing neurites (mean ± SEM; three independent experiments). ***p = 0.00036.

(F) The longest axon on about 300 neurons per condition was measured (mean ± SEM).

(G–L) Neurons were cultured on laminin-coated coverslips containing an orientation grid to allow relocalization of the same neurite over time. Brief electric pulses were applied to the neurons through two electrodes placed in the culture dish.

(G) Illustration of the stimulus pattern applied to the cells.

(H) Whole-cell recording showing that cells fire action potentials in response to the stimulation paradigm. Only neurites growing during the first hour without any stimulus (0 to 1 hr) were further evaluated for their growth under no stimulation (I), stimulation (J), stimulation in the presence of 1 μM tetrodotoxin (TTX) (K), and in the presence of TTX alone for another hour (1 to 2 hr). Filled arrowhead points to the tip of the process, and its distance to the open arrowheads indicates the growth of the neurite. The identity of the neurite was confirmed at the end of the experiment by fixing and staining the cells with Tuj-1 antibody. Scale bar represents 10 μm.

(L) Data analysis comparing axon growth rates between the two time periods (ratio = 0–1 hr/1–2 hr) for individual neurites (mean ± SEM; n > 20 neurites for each condition). Stim ***p = 5.3 × 10^{-6}; KCl ***p = 2.3 × 10^{-5}.

which are proprioceptive afferents [21] whose central axons project into the dorsal column. These neurons are electrically silent in culture (see Figure S1D available online); they are deprived of their innervation targets, and they do not form synaptic connections with other DRG neurons. In order to mimic an electrically active state, we followed two approaches: chronic depolarization by high K^+ and electric field stimulation.

In the first approach, we depolarized neurons by incubating them in culture media containing 40 mM KCl and compared neurite outgrowth to control neurons 3 days after plating. In response to high K^+, the membrane potential of DRG neurons shifted from −56 ± 2 mV to −9 ± 1 mV (Figures S1A and S1D). After 3 days in high K^+, the cells remained "clamped" at a depolarized potential of −17 ± 1 mV. The resting membrane potential was rapidly reestablished after high K^+ washout (Figures S1B–S1D). Chronic membrane depolarization resulted in a 2.5-fold reduction in the number of neurons with neurites (Figures 2A, 2B, and 2E) and a concomitant decrease in their neurite length (Figure 2F). The effect of depolarization was not caused by changes in medium osmolarity, because neurons treated with 40 mM NaCl showed an axonal growth pattern indistinguishable from control neurons (Figure 2C). Cells cultured under depolarizing conditions remained viable. Depolarized cells that were returned to control medium for 3 additional days showed extensive neurite growth and a high number of neurites (Figure 2D). Thus, depolarization inhibits axon outgrowth in cultured adult DRG neurons.

To study whether more physiological levels of activity also inhibit growth, we next assessed the effect of electrical stimulation in growing neurites. To stimulate neurons to fire action potentials (APs), we applied brief trains of voltage pulses at 20 Hz interleaved with 5 min off periods (Figure 2G), resembling sensory stimulus-evoked activity [22]. Through separate patch-clamp experiments, we validated that the stimuli were suprathreshold, i.e., neurons fired APs in response to the stimulation paradigm (Figure 2H). Elongation of individual neurites was evaluated during periods of electrical silence and activity. Whereas neurites grew at a constant growth rate when no stimulation was applied to the cells (Figures 2I and 2L), neurite

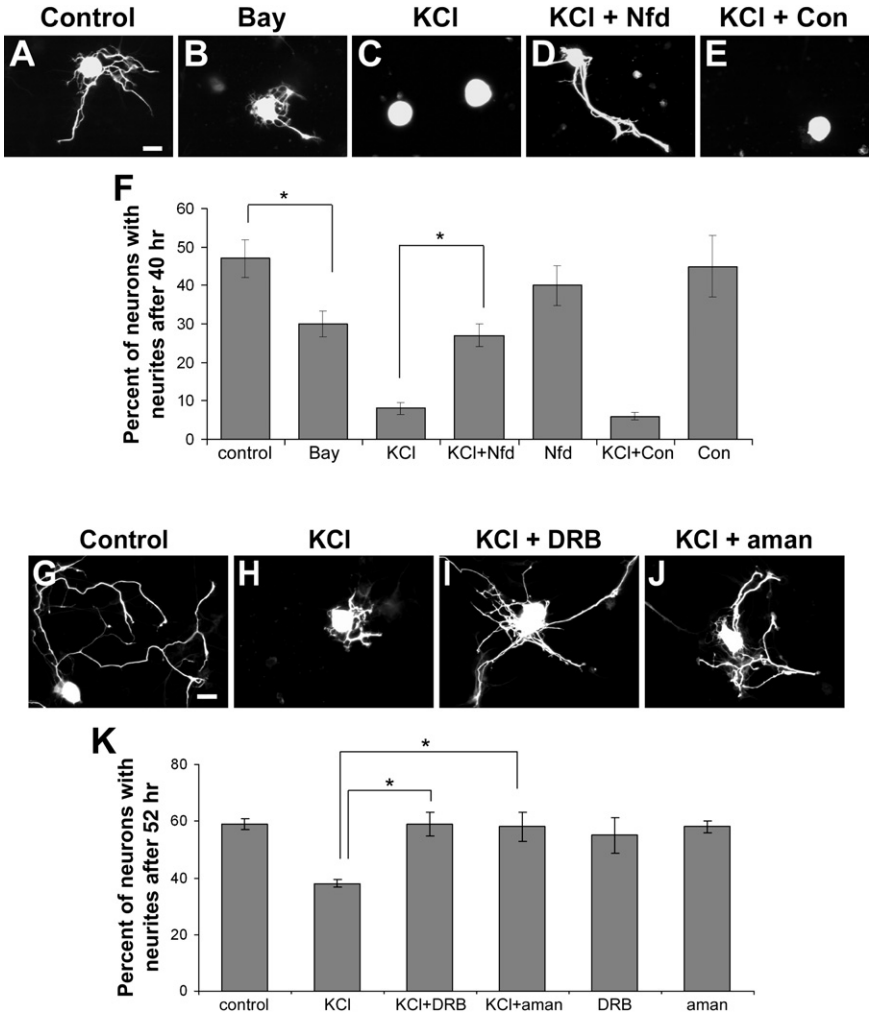


Figure 3. Axon Growth Inhibition Is Mediated by L-Type Ca^{2+} Current and Involves the Transcription of Growth Inhibitory Genes

(A–F) Dissociated neurons were grown for 40 hr on poly-lysine in the conditions as indicated. To determine the effect of L-type Ca^{2+} current activation, we grew neurons in the absence (A) or presence (B) of the L-type Ca^{2+} channel agonist Bay K 8644 (10 μM). *p = 0.031. The contribution of the different voltage-gated Ca^{2+} currents to the inhibitory effect of neuron depolarization was assessed using specific channel blockers. Neurons were depolarized with 40 mM KCl alone (C) or in the presence of L-type channel blocker nifedipine (10 μM) (D) or general blocker for the other high-voltage activated Ca^{2+} currents *w*-conotoxin MVIIC (200 nM) (E).

(F) Quantification of neurite formation under the different treatments (mean ± SEM; three independent experiments). *p = 0.013.

(G–K) Cells were grown for 40 hr on poly-lysine in control media and thereafter in the mentioned media for a period of 12 hr.

(G) Neurons cultured for 52 hr in control media.

(H–J) Neurons incubated in 40 mM KCl for the last 12 hr in the absence (H) or presence of gene transcription blockers, 40 μM DRB (I) (*p = 0.032) or 500 nM α-amanitin (J) (*p = 0.049). Scale bar represents 25 μm.

(K) Quantification of neurite outgrowth (mean ± SEM; three independent experiments).

growth was immediately halted with the onset of electrical stimulation (Figure 2J). Cessation of growth could be largely prevented by the addition of the Na^+ channel blocker tetrodotoxin (TTX) (Figure 2K), showing that growth inhibition depends on AP firing. TTX alone had no effect on axon growth (Figure 2L). We additionally confirmed that high K^+ directly affected axon elongation (Figure 2L). Taken together, these findings demonstrate that electrical activity impairs axon initiation and elongation in adult DRG neurons.

Axon Growth Inhibition Is Mediated by L-Type Voltage-Gated Ca^{2+} Channels and Depends on Gene Transcription

To gain insight into the downstream effectors of electrical activity, we looked for factors that could counterbalance its inhibitory action. Activity-induced membrane depolarization triggers influx of Ca^{2+} ions, mainly via voltage-gated calcium channels, leading to an increase in $[Ca^{2+}]_i$. We verified that neurons chronically depolarized for 3 days still showed levels of $[Ca^{2+}]_i$ higher than those in control neurons (Figure S1E). We therefore examined growth of depolarized neurons in the presence of specific voltage-gated Ca^{2+} channel blockers. Five different types of VGCC have been described to coexist in DRG neurons, T-type, P-type, Q-type, N-type, and L-type, differing in their kinetics and pharmacological properties [23–25]. The low-voltage-activated T-type current is rapidly blocked by keeping the cells at a depolarized membrane potential of about −20 mV, which occurs in the presence of high K^+ [26]. Thus, only the high-voltage-activated Ca^{2+} currents (P-type, Q-type, N-type, and L-type) are likely to mediate the effect of electrical activity. Growth inhibition persisted in the presence of ω-conotoxin MVIIC, which blocks P-type, Q-type, and N-type channels (Figures 3C, 3E, and 3F), suggesting that these channels are not involved in inhibition induced by high K^+. In contrast, about 50% of growth inhibition was prevented by blocking the L-type channels with nifedipine (Figure 3D). Conversely, L-type channel activation by the agonist Bay K impaired growth (Figures 3A and 3B). Neither conotoxin nor nifedipine alone affected axon growth (Figure 3F). These data indicate that the inhibitory effect of electrical activity on axon growth depends, at least in part, on L-type channel activation.

Previous studies suggest that L-type Ca^{2+} channels regulate different cellular functions by modulating gene expression [27]. Therefore, we hypothesized that depolarization might inhibit axon growth by inducing the expression of growth inhibitors. This scenario implies that growth under depolarizing conditions can be rescued when gene transcription is blocked. Because a discrete initial period of de novo gene transcription is necessary to support growth, we assessed the role of growth inhibitors at a later time point in culture, when neurons are able to grow for a period of 12 hr in the absence of transcription [3] (Figure 3K). Therefore, blockers of gene transcription were added to cells for 12 hr after an initial period of 40 hr in culture at the same time at which depolarization was evoked by 40 mM KCl. When gene transcription was blocked during this interval by DRB, an inhibitor of RNA polymerase II, depolarization did not inhibit growth

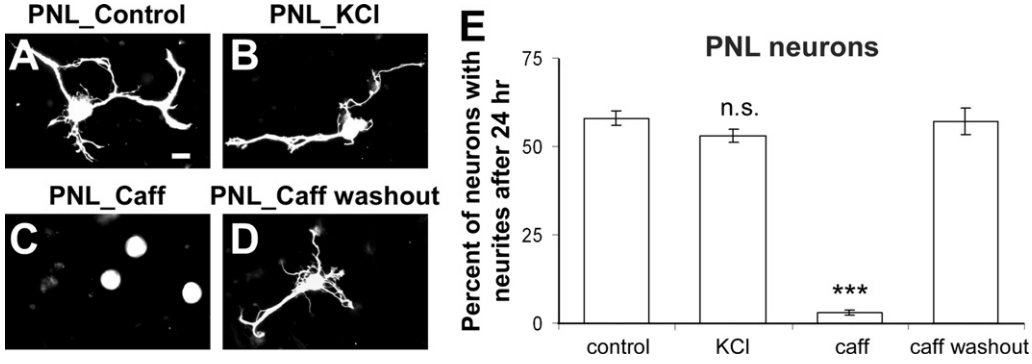


Figure 4. PNL Neurons Show a Different Response to Neuron Depolarization

(A–E) Neurons were isolated from rats subjected to peripheral injury 3–7 days before culture and plated on poly-lysine for 1 day. Cells grown in control (A) and 40 mM KCl media (B) show extensive neurite formation, whereas cells cultured in 10 mM caffeine media (C) exhibit fewer processes.

(D) Neurons cultured in media containing 10 mM caffeine for 1 day and subsequently in control media for an additional day. Scale bar represents 25 μm.

(E) Quantification showing the percent of neurons forming neurites (mean ± SEM; three independent experiments). ***$p = 2.1 \times 10^{-6}$; not statistically different (NS, $p = 0.063$).

(Figures 3G–3I and 3K). A similar result was obtained using another inhibitor of RNA polymerase II, α-amanitin (Figure 3J). These findings suggest that electrical activity inhibits growth in a transcription-dependent manner.

Peripheral Nerve Lesioned Neurons Are Not Inhibited by Neuron Depolarization

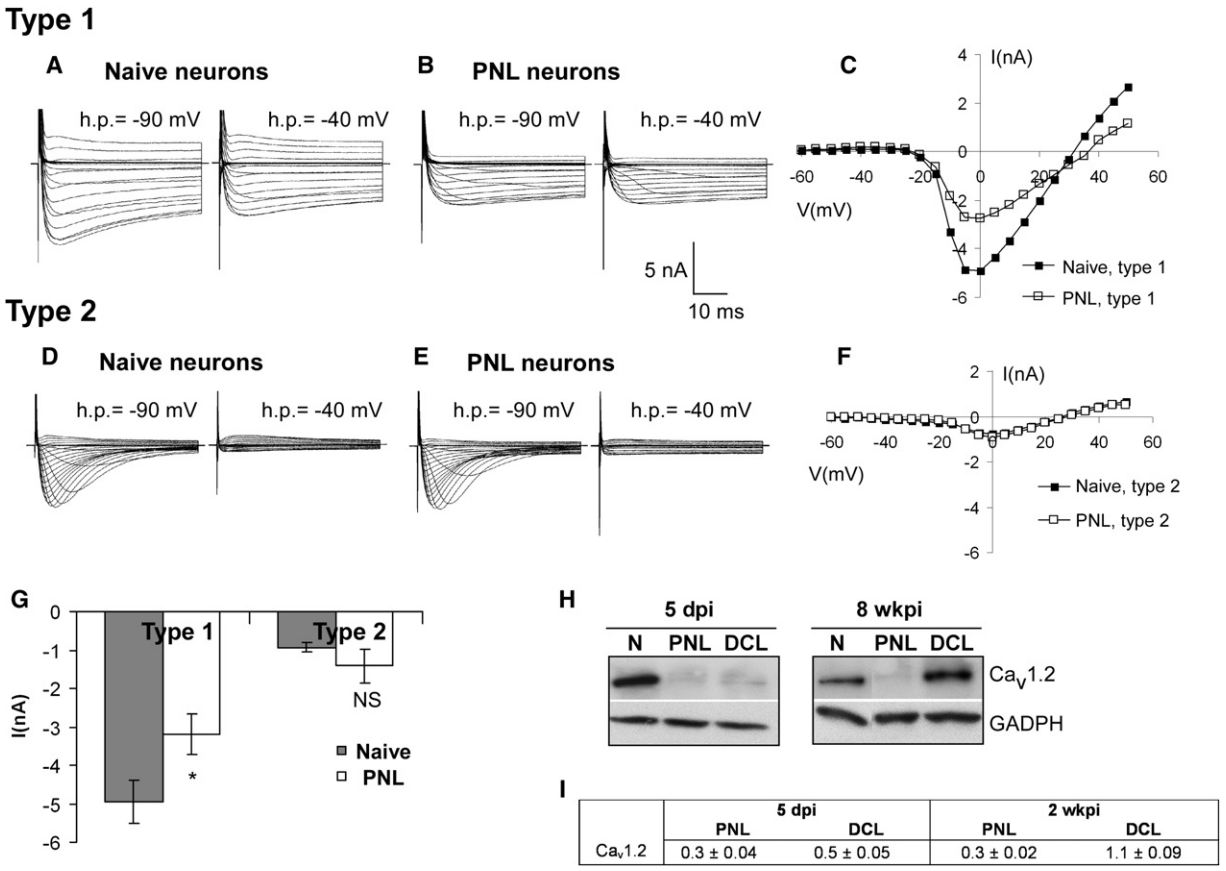
A peripheral lesion remarkably enhances the growth competence of the affected DRG neurons. Whereas naive (unlesioned) neurons require some days to extend neurites and are quiescent during the first 24 hr after plating, neurons subjected to a peripheral injury 3–7 days before isolation show robust neurite growth already at 24 hr in culture [3, 28].

To further study whether electrical activity is determinant for the enhanced growth competence seen in adult DRG neurons after peripheral lesion, we explored changes in the electrophysiological properties of PNL neurons that might allow them to circumvent inhibition and promote growth. We observed that, unlike naive neurons (Figures 2A and 2B), neurite growth of PNL neurons is not impaired by depolarization (Figures 4A, 4B, and 4E). Therefore, we next sought to identify the level on the inhibitory pathway at which insensitivity to electrical activity is conferred. One possible cause for insensitivity could be altered plasma membrane properties triggered by the lesion, specifically a reduced Ca^{2+} influx. To assess this possibility, we asked whether an increase in $[Ca^{2+}]_i$ would inhibit growth on PNL neurons. To this end, we incubated neurons in caffeine to induce Ca^{2+} release from intracellular stores and, thereby, an elevation in $[Ca^{2+}]_i$. All cells responded to caffeine treatment with a steady increase in $[Ca^{2+}]_i$, in that 70% of them also generated periodic $[Ca^{2+}]_i$ oscillations (Ca^{2+} sparks) [29] (Figures S2F and S2G; Movie S1; Movie S2). These $[Ca^{2+}]_i$ oscillations remained during long-term treatment in most of the neurons for at least 24 hr and were reduced after caffeine washout (Figure S2G; Movie S3; Movie S4). We observed that incubation in caffeine substantially decreased outgrowth on PNL neurons (Figure 4C). Because caffeine-treated neurons grew neurites after the washout (Figure 4D), the inhibitory effect on axon growth is likely to be associated to an elevation in $[Ca^{2+}]_i$, and not to a decrease in cell viability. Notably, other pathways activated by caffeine, e.g., increase in intracellular cyclic AMP level, which was shown to enhance growth of adult DRG neurons [28, 30], were not sufficient to counteract the inhibitory effect. Thus, these findings suggest that insensitivity to electrical activity after peripheral injury is conferred by a reduced Ca^{2+} influx.

To test this idea, we loaded DRG neurons with the Ca^{2+} indicator Oregon Green BAPTA-1 AM and measured the elevation in $[Ca^{2+}]_i$ in response to a pulse of high K^+. Depolarization triggered a smaller $[Ca^{2+}]_i$ elevation in PNL neurons as compared with naive neurons (Figures S2A–S2E). This result shows that intracellular Ca^{2+} levels are altered after a peripheral injury, leading to a smaller Ca^{2+} signal upon depolarization. Overall, these data suggest that a peripheral lesion triggers electrophysiological changes that allow adult DRG neurons to escape the growth inhibitory effects of electrical activity.

L-Type Ca^{2+} Channels Are Downregulated after Peripheral Injury

Because we showed that L-type Ca^{2+} channel activity mediates growth inhibition, we next assessed for a possible reduction of this particular current after peripheral lesion. Thus, we performed voltage-clamp recordings in dissociated DRG neurons to compare L-type current amplitudes in naive and PNL neurons. Ca^{2+} currents mediated by T-type and L-type channels were isolated by recording in an extracellular solution free of Na^+ and K^+ ions and by additionally blocking P-type, Q-type, and N-type Ca^{2+} currents with ω-conotoxin MVIIC. Currents were evoked by a series of 50 ms voltage steps from a holding potential of either −90 mV or −40 mV. We observed the presence of two different cell types in culture, distinguishable by their current profile evoked from a holding potential of −90 mV, as previously described [31]. Type 1 neurons possessed only a high-voltage-activated, slowly inactivating L-type Ca^{2+} current (Figures 5A–5C), representing approximately 70% of the neuronal population in cell culture. Instead, type 2 neurons showed a low-voltage-activated, fast inactivating T-type Ca^{2+} current and only a small L-type component (Figures 5D–5F). They represented approximately 30% of the neuronal population. Therefore, differences in L-type current amplitude between naive and PNL neurons were evaluated separately for type 1 and type 2 cells. By holding the neurons at −40 mV to inactivate the T-type currents and then stepping to the various test potentials, we isolated L-type currents (Figures 5A, 5B, 5D, and 5E, right traces). We found that peripheral injury significantly reduced



	5 dpi		2 wkpi	
	PNL	DCL	PNL	DCL
$Ca_v1.2$	0.3 ± 0.04	0.5 ± 0.05	0.3 ± 0.02	1.1 ± 0.09

Figure 5. L-Type Ca^{2+} Currents Are Reduced after Peripheral Nerve Lesion

Voltage-clamp recordings were performed in control and PNL neurons in the presence of 200 nM *w*-conotoxin MVIIC.

(A, B, D, and E, left traces) Neurons were classified as type 1 or type 2 based on their current profile evoked from a holding potential of −90 mV. Type 1 neurons feature only slow-inactivating high-threshold L-type currents, whereas type 2 neurons feature both L-type and fast-inactivating low-threshold T-type currents. In control cells, 71% (15 of 21) were type 1 and 29% (6 of 21) were type 2. Type frequency was not affected by peripheral injury. To compare L-current amplitude between control and PNL neurons, we also recorded cells from a holding potential of −40 mV to functionally remove the fast-inactivating T-type component.

(A and B, right traces, and C) Representative traces of type 1 neurons showing that L-type current amplitude is reduced after peripheral lesion.

(D and E, right traces, and F) Representative traces of type 2 neurons showing that L-type current amplitude is not affected by peripheral lesion. Note that type 2 cells have a characteristic small L-type current. (C) and (F) plots show the current-voltage (I-V) relationship for the four cells presented here.

(G) Average of L-type current peak amplitudes recorded from control and PNL neurons (mean ± SEM; 21 neurons per condition). *p = 0.034; not statistically different (NS, p = 0.3).

(H and I) Changes in $Ca_v1.2$ protein (H) and gene expression (I) levels in the dorsal root ganglia (DRG) neurons after peripheral or central axotomy.

L-type current amplitude in type 1 neurons (Figures 5A–5C and 5G). The small L-type component observed in type 2 neurons was unaltered by injury (Figures 5D–5F). These data demonstrate that the L-type current amplitude is decreased in the majority of PNL neurons.

To test whether the decrease in L-type current is caused by a downregulation at the protein or gene expression level, we next compared the amount of the pore-forming subunit of the L-type channel protein and mRNA. Two isoforms, $Ca_v1.2$ and $Ca_v1.3$, can constitute the pore of L-type channels, but $Ca_v1.2$ is predominantly expressed in medium- and large-diameter DRG neurons [32] (Figure S3A). Western blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed lower $Ca_v1.2$ protein and gene expression levels, respectively, in PNL ganglia (Figures 5H and 5I). This reduction was observed both at early and late time points after peripheral injury. Parallel to the analysis on PNL ganglia, we also checked for $Ca_v1.2$ expression on ganglia isolated from DCL rats. Unexpectedly, we observed that $Ca_v1.2$ expression was downregulated at 5 days after DCL, but not at later time points (Figures 5H and 5I). These data suggest that peripheral nerve lesion induces a persistent downregulation of $Ca_v1.2$ protein and gene expression in DRG neurons, whereas dorsal column lesion leads to biphasic change in the levels of $Ca_v1.2$ expression, namely its early downregulation and subsequent return to normal levels. Consistent with $Ca_v1.2$ downregulation enhancing growth, it has been demonstrated that acutely injured DCL neurons show axon growth patterns intermediate between naive and PNL neurons [3].

Taken together, these findings show a persistent decrease in L-type channels in the cell membrane of PNL neurons that might endow PNL neurons with enhanced growth competence.

Ablation of $Ca_v1.2$ Channel Subunit in the Nervous System

We showed that electrical activity inhibits axon growth through activation of the L-type channels. Moreover, conditioned DRG neurons show a downregulation of L-type

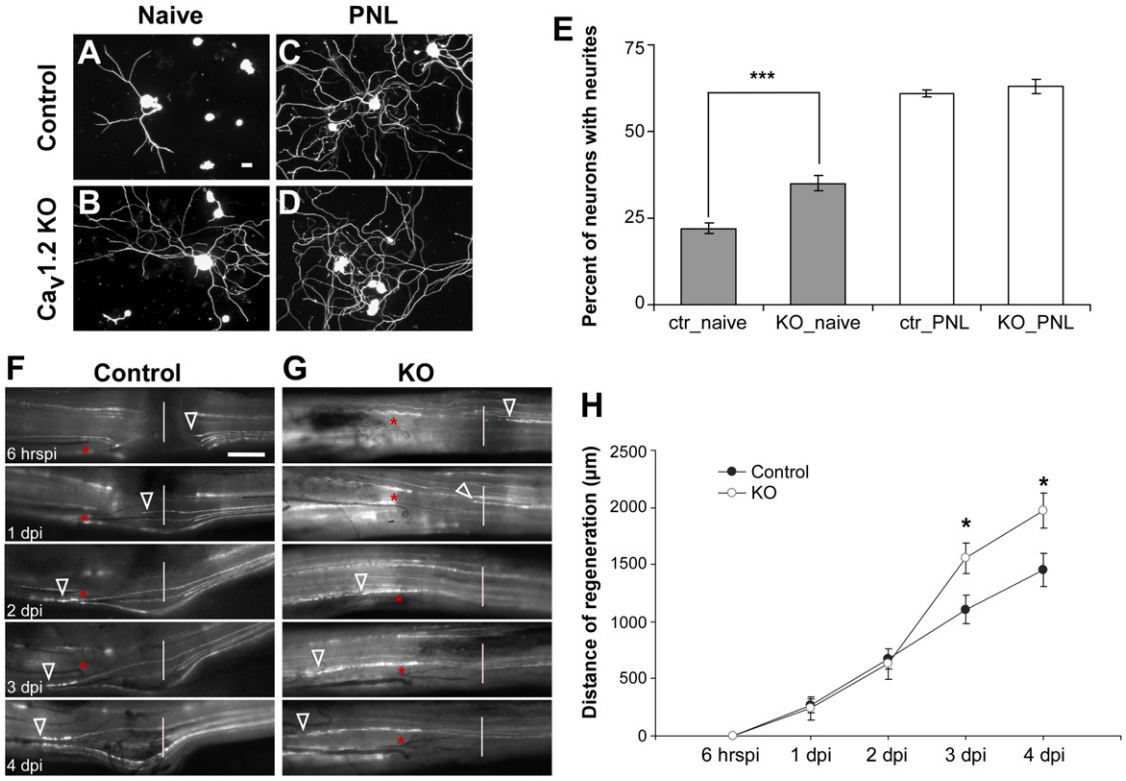


Figure 6. Lack of L-Type Ca^{2+} Current Is Sufficient to Boost the Growth Ability of Adult DRG Neurons

(A and B) Neurons were isolated from $Ca_v1.2$ knockout (KO) and heterozygous (control) adult mice and cultured on laminin for 18 hr.

(C and D) Neurons subjected to peripheral injury 5 days before isolation.

(E) Quantification showing that loss of L-type current potentiates neurite formation in DRG neurons (mean ± SEM; n = 9 KO mice). ***p = 0.00017.

(F–H) In vivo imaging of regenerating axons in the sciatic nerve. The proximal tips of the injured axons were imaged over 4 days after sciatic nerve crush in control (F) and $Ca_v1.2$ KO (G) mice. Vertical line indicates the lesion site, open arrowheads indicate the axonal tips. Veins served as landmarks (*) to define the positioning of the axonal tips relative to the lesion site. Scale bar represents 500 µm.

(H) Quantification of axon regeneration at the different imaging time points. The average distance of regeneration of injured axons was calculated for each animal (distance values calculated relative to the 6 hours post injury (hrspi) time point; mean ± SEM; n = 10 mice per condition). *p = 0.025 at 3 days per injury (dpi); *p = 0.024 at 4 dpi.

channels. This raises the question of whether the absence of this channel type would be sufficient to promote axon growth of adult DRG neurons.

We utilized the Cre recombinase system using Nestin-Cre transgenic mice [33] to create a mouse line with an inactivation of the *CACNA1C* gene ($Ca_v1.2$) in the whole CNS. $Ca_v1.2^{L1/L2}$ Nes-$Cre^{+/tg}$ mice, which we call here $Ca_v1.2$ knockout (KO) mice, were viable and exhibited normal life expectancy, body weight, and breeding. Their brains did not show any obvious morphological abnormalities. Mating with the reporter rosa 26 line showed that Nestin-Cre mice have a strong Cre recombinase activity in DRG neurons (Figure S3B). Western blot analysis confirmed that $Ca_v1.2$ protein was absent from DRGs, as well as from the whole brain of KO animals (Figures S3C and S3D). There were no compensatory changes of the $Ca_v1.3$ protein level in the $Ca_v1.2$ KO DRG, as demonstrated by semiquantitative RT-PCR (Figure S3E).

$Ca_v1.2$ KO Neurons Show Enhanced Outgrowth in Cell Culture

We then investigated whether genetic inactivation of $Ca_v1.2$ L-type Ca^{2+} channels would be sufficient to promote axon growth in adult DRG neurons. To this end, we isolated naive neurons from control and $Ca_v1.2$ KO animals. Because mouse adult DRG neurons do not grow well on poly-lysine, we cultured them on laminin. Because of the enhanced growth on laminin [3], we evaluated outgrowth after 18 hr in culture. There was no obvious effect of the loss of $Ca_v1.2$ in neuronal survival (Figure S3F). KO neurons exhibited a more robust outgrowth in comparison to littermate control neurons (1.6-fold increase; Figures 6A, 6B, and 6E; Figure S3G), indicating that loss of L-type channel activity is sufficient to enhance the growth ability of adult DRG neurons. Because neurons previously subjected to a peripheral axotomy grew 2.8-fold more than control neurons (Figures 6C and 6E), we conclude that ablating $Ca_v1.2$ induces approximately 30% of the growth enhancement seen in PNL neurons.

We next assessed whether loss of L-type channels is involved in the mechanism enhancing axon growth after peripheral lesioning. To this end, we performed conditioning lesions in the KO animals. If L-type channel downregulation and peripheral lesioning would be independent events, one would expect that their effects should be cumulative. Instead, if L-type channel downregulation is part of the conditioning paradigm, one would expect that there is no additive effect. Indeed, we found that conditioned KO neurons grew to a similar extent as the conditioned control neurons (Figures 6C–6E). These findings suggest that reduction of L-type Ca^{2+} channel activity contributes to the effect of conditioning lesion on growth competence.

$Ca_v1.2$ KO Mice Show Enhanced Peripheral Nerve Regeneration

Because the loss of L-type Ca^{2+} channels promotes axon outgrowth in dissociated DRG neurons, we next explored whether it is sufficient to enhance axon regeneration in vivo. A conditioning lesion performed 1–2 weeks before sciatic nerve crush increases axonal outgrowth from the injured axons [34]. Therefore, enhanced regeneration potential can also be assessed in the PNS. We investigated peripheral axon regeneration in control and $Ca_v1.2$ KO mice by in vivo imaging. To this end, we crossed the $Ca_v1.2$ KO with the GFP-M mouse line [35] to generate transgenic animals expressing GFP in a small group of neurons with a control or $Ca_v1.2$ KO background. We performed sciatic nerve crush in control and KO mice and imaged single GFP-labeled axons over a period of 4 days post injury. After an initial phase of axonal retraction, the majority of injured axons started regenerating and crossed the lesion site by day 1 (Figures 6F and 6G). We found that regenerating axons in $Ca_v1.2$ KO mice grew farther than those of control mice (1973 ± 152 μm in KO mice compared to 1452 ± 147 μm in control mice at 4 days post injury; Figure 6H). Our findings led us to conclude that loss of L-type Ca^{2+} channels improves axon regeneration in vivo.

Discussion

Embryonic neurons grow their axons over long distances to connect with their appropriate targets. As the neuron matures, the genetic program associated with axon growth is gradually suppressed. In adult primary sensory neurons, however, a conditioning peripheral lesion reactivates the genetic program to promote axon regeneration [8]. The cellular basis of this growth competence switch remains poorly understood. Our study provides evidence that electrical activity is a signal suppressing axon growth competence through L-type Ca^{2+} channels. We propose that electrical activity is the negative target-derived signal that is deactivated through conditioning.

Electrical Activity as the "Intrinsic Negative Signal" for Axon Regrowth

Electrical activity modulates axon growth in a cell-type-dependent manner during neuronal development. Whereas it potentiates axon growth in rat retinal ganglion cells [14] and sympathetic motor neurons [15], it stops axon growth in mouse primary sensory neurons [16, 36]. It has been previously suggested that electrical activity acts as a physiological sensor for the embryonic sensory neuron, signaling its connection to the peripheral target cell [19, 37]. Axon stalling in response to electrical input may be a key feature of DRG neurons attributable to their unique structure. Although other neuronal cell types receive input largely via their dendrites, the receiving element in DRG neurons is their peripheral axon.

Interestingly, our results showed that electrical activity inhibits axon growth also in adult DRG neurons. Because the stimulation protocol used in our experiments resembles the normal AP firing pattern of adult sensory neurons [22], our findings support the initial hypothesis that normal levels of activity experienced by functional adult DRG neurons can indeed suppress growth. Therefore, the mechanism of growth inhibition by electrical activity seems to be conserved throughout adulthood, controlling the regenerative state of sensory neurons. Our in vivo recording data showed that only a peripheral lesion silences primary sensory neurons, setting them in a growth-competent state. Although it has been reported that some DRG neurons may generate spontaneous activity after peripheral deafferentiation, this occurs at later stages and in a very small percentage of the neurons [38–40], as our results confirmed.

Electrical stimulation is currently regarded as a general positive regulator of axon regeneration, independently of the cell type affected [41]. For example, application of weak electrical fields (about 10 mV/mm) at the site of injury has been described to improve recovery of the propriospinal intersegmental reflex in guinea pigs [42]. A more recent study suggested that electrical stimulation of the sciatic nerve promotes regeneration of the central injured sensory fibers [43]. Importantly, the weak electrical fields are applied to the injured tips, and the outcome is strictly dependent on the direction of the applied field. The effects have been associated with local cytoskeleton remodeling [44] and reduction of the initial cationic current that enters the tip of the axon, thereby preventing massive retraction [45]. By contrast, in our experiments, we applied a strong electrical field to DRG cell bodies to mimic the physiological activity experienced by adult DRG neurons. Consistently, our analysis indicates a global Ca^{2+} signal that elicits a nuclear response, leading to long-term modifications of the genetic program of the cell. Therefore, this apparent discrepancy may be due to differences in the stimulation paradigm. Different spatiotemporal characteristics of the changes in $[Ca^{2+}]_i$ can activate different downstream targets and generate diverse cellular responses [17].

Our study provides the first evidence, to the best of our knowledge, that electrical activity represses the growth competence of adult DRG neurons. We propose that this mechanism applies specifically to primary sensory neurons and that it needs to be relieved to allow axon regeneration.

Downstream Effectors of Growth Inhibition Elicited by Electrical Activity

Our studies implicate the L-type Ca^{2+} channel as a mediator of axon growth inhibition by electrical activity, because the block of L-type channels prevented axon growth inhibition in depolarized neurons to a large extent. We envision that Ca^{2+} influx through L-type channels further stimulates the release of Ca^{2+} from internal stores. Consistently, we found that axon growth in conditioned neurons ceases in the presence of caffeine, which is known to induce Ca^{2+} sparks arising from Ca^{2+} release from, and reuptake into, internal stores [29]. Similar Ca^{2+} sparks can also be evoked in response to membrane depolarization [46].

The involvement of L-type channels in this process raises the interesting possibility that electrical activity may interfere with the genetic program controlling growth in sensory neurons, because this particular channel type is known to regulate gene transcription in several ways. Not only does Ca^{2+} influx through L-type channels regulate the activity of several Ca^{2+}-dependent transcription factors and transcriptional repressors [27], but also the carboxyl terminus of L-type channels can translocate to the nucleus and act itself as a transcription factor (CCAT) [47]. In this context, our demonstration that transcription blockers abrogate the inhibitory effect of depolarization suggests that electrical activity may lead to the expression of either intrinsic growth inhibitory factors or transcriptional suppressors of growth-promoting proteins.

In addition to a long-term effect at the gene transcriptional level, electrical activity may also have a short-term effect in the growth of adult DRG neurons in culture. The electrical field

stimulation experiments show that growth inhibition is visible within 1 hr of stimulation. Although immediate early genes might account for this fast response, it is possible that other mechanisms operate at this early time period. For instance, short-term effects of electrical activity may involve changes in cytoskeletal structure and dynamics that alter growth cone behavior and axon extension [17].

In summary, electrical activity and L-type Ca^{2+} current could be part of the same intracellular pathway that inhibits axon growth: neuronal activity induces membrane depolarization, which leads to the opening of voltage-gated calcium channels and influx of Ca^{2+}, which in turn inhibits growth in a transcription-dependent manner.

A Peripheral Lesion Reduces Ca^{2+} Influx through L-Type Ca^{2+} Channels

Immediately after axonal injury, there is a large Ca^{2+} influx from the extracellular space through the cut end, which is important for rapid membrane resealing and growth cone formation. However, this acute elevation in $[Ca^{2+}]_i$ turns back to resting levels within a few minutes [48]. An interesting finding from our studies is that, at a longer term, a peripheral lesion leads to a reduction in Ca^{2+} influx. By semiquantitative Ca^{2+} imaging analysis, we found that PNL neurons show a smaller $[Ca^{2+}]_i$ elevation in response to high K^+ than naive neurons. By voltage-clamp analysis, we showed that L-type channel activity is reduced in PNL neurons when compared to naive neurons. Our western blot and RT-PCR analysis on the $Ca_v1.2$ expression confirmed a persistent downregulation of $Ca_v1.2$ after peripheral lesion. These results are further supported by previous reports showing that Ca^{2+} entry is downregulated in DRG neurons after peripheral injury in rats [31, 49, 50]. Intriguingly, we also found that $Ca_v1.2$ expression was downregulated during the acute phase of dorsal column lesion but returned to normal levels during the chronic phase. It is worth noting that injury induces an early accumulation of intracellular Ca^{2+} and that a significant increase in $[Ca^{2+}]_i$ can be cytotoxic. Hence, it may be that $Ca_v1.2$ downregulation at early stages after injury is part of a more general program elicited to prevent cell death during a critical period characterized by a strong inflammatory response, as previously suggested by others [51]. The return to normal levels of $Ca_v1.2$ expression during the chronic phase, in which the spinal cord shows attempts to repair, may contribute to the failure of axon regeneration on primary sensory neurons after dorsal column lesion.

Our results establish a link between a specific electrophysiological change—the reduction of L-type channel current—and axon growth competence. It therefore corroborates with the lack of electrical input after peripheral injury to provide further evidence that electrical silencing is necessary for axon growth. We provided three additional lines of evidence for the involvement of the L-type Ca^{2+} channels in the conditioning lesion effect: first, genetic inactivation of this channel type is sufficient to enhance the growth ability of naive adult DRG neurons in culture; second, genetic inactivation of the $Ca_v1.2$ channels does not further improve growth on PNL neurons, suggesting that the lack of L-type channels is a component of the conditioning effect; and third, loss of $Ca_v1.2$ alone is sufficient to improve axon regeneration after sciatic nerve crush. Although $Ca_v1.2$ KO neurons regenerate better than control neurons after PNS injury, we did not observe improvement of axon regeneration after CNS injury (data not shown). It is important to note that conditioning is a multifactorial process whereby several factors act together to promote robust regeneration in the CNS environment. Although loss of $Ca_v1.2$ enhances axon growth competence, it is not sufficient to enhance regeneration in the CNS, probably because neurons cannot overcome the inhibitory extracellular environment. Interestingly, a recent study in lamprey (which, unlike higher vertebrates, regenerates spontaneously) shows that there is a significant reduction in mRNA levels of voltage-gated Ca^{2+} channels during the regeneration phase [52].

It is a surprising result, though, that L-type voltage-gated Ca^{2+} channels are downregulated after peripheral axotomy, because the lack of sensory input alone would be enough to render these channels inactive. A possible explanation for a coordinated downregulation of Ca^{2+} influx after injury is the fact that several other pathways converge to Ca^{2+} to inhibit axon regeneration after injury, notably those targeted by extracellular growth inhibitory molecules. For instance, large rises in intracellular $[Ca^{2+}]$ are necessary to mediate growth cone retraction upon contact with the myelin-associated proteins MAG [53] and Nogo [54]. Therefore, reduction of Ca^{2+} influx through L-type channels might be important to enhance the intrinsic growth competence and to prevent inhibition by extracellular molecules, both relevant factors in the context of spinal cord injury. Another possible explanation is that loss of electrical activity per se downregulates $Ca_v1.2$ expression. The existence of a feedback loop between electrical activity and expression of ion channels has been previously shown in *Xenopus* embryonic neurons [11].

Conclusions

The data presented here provide evidence that electrical activity is an intrinsic negative signal suppressing the growth competence of adult primary sensory neurons. We propose that electrical silencing following peripheral deafferentiation contributes to the conditioning effect eliciting axon growth competence. In developing strategies for spinal cord regeneration, it may be important to consider electrical silencing as an intrinsic factor that can act complementarily and synergistically with extracellular factors to release the preimposed "brake" and prime neurons to grow.

Experimental Procedures

The experimental procedures are described in detail in the Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and four movies and can be found with this article online at doi:10.1016/j.cub.2010.05.055.

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