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Neonatal vincristine administration modulates intrinsic neuronal excitability in the rat DRG and spinal dorsal horn during adolescence

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Abstract

Our recent work has shown that early life administration of vincristine (VNC), commonly used to treat pediatric cancers, evokes a delayed mechanical pain hypersensitivity in rats that emerges during adolescence. However, the underlying mechanisms remain unclear, as nothing is known about how neonatal VNC treatment influences peripheral and central nociceptive processing at the cellular level. Here we used *in vitro* intracellular microelectrode and whole-cell patch clamp recordings to evaluate the consequences of early life VNC administration on the intrinsic membrane properties of adolescent dorsal root ganglion (DRG) and spinal superficial dorsal horn (SDH) neurons. The results demonstrate that VNC treatment increased the prevalence and rate of repetitive firing in both large- and medium-diameter sensory neurons, while reducing spike discharge in small-diameter neurons, in comparison to vehicle-treated littermate controls. In contrast, passive membrane properties and axonal conduction velocities were similar between experimental groups across all classes of primary afferents. Within the adolescent SDH, neonatal VNC exposure significantly enhanced the intrinsic membrane excitability of lamina I spino-parabrachial neurons, as evidenced by a decrease in rheobase and elevation of repetitive firing frequency compared to controls. Meanwhile, putative interneurons within lamina I exhibited a reduction in repetitive action potential discharge following early life chemotherapy. Collectively, these findings suggest that neonatal VNC treatment evokes cell type-specific changes in intrinsic excitability at multiple levels of the ascending pain pathway. Overall, this work lays an essential foundation for the future exploration of the ionic mechanisms which drive chemotherapy-induced chronic pain in children and adolescents.

Abstract

Vincristine treatment during early life evokes cell-type dependent alterations in the intrinsic membrane excitability of primary afferent and spinal dorsal horn neurons during adolescence.

1. Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a major adverse effect of many chemotherapeutic agents including vincristine (VNC), commonly used to treat the most prevalent childhood malignancy, Acute Lymphoblastic Leukemia (ALL). CIPN is characterized by sensory disturbances such as paresthesias and dysesthesias in the hands and feet, thereby negatively impacting the overall quality of life in pediatric and adult cancer patients [19,33,68]. CIPN recovery time and the predominance of sensory vs. motor symptoms may differ between age groups [29,43]. Furthermore, VNC administration to neonatal rats evokes an age-dependent mechanical pain hypersensitivity that emerges during adolescence and persists into adulthood [59]. However, the underlying mechanisms remain unclear, as little is known about the influence of early life VNC on the function of neurons within the developing pain pathway.

CIPN alters the intrinsic excitability of several classes of adult DRG neurons, as evidenced by abnormal spontaneous discharge and enhanced responsiveness to electrical, mechanical, and thermal stimuli [42,57,64,73,79]. The observed effects on multiple subtypes of sensory neurons are consistent with clinical reports suggesting that patients with CIPN also exhibit impaired thermal discrimination and compromised motor function, indicating a possible involvement of small-diameter fibers and proprioceptors in addition to large-diameter mechanoreceptors [7,18,43,51]. Nonetheless, it should be noted that peripheral nerve injury or inflammation can evoke sensory neuron dysfunction in an age-dependent manner [5,16,28,70]. This suggests that the known effects of chemotherapy in adults cannot be easily extrapolated to adolescence, and highlights the need to better understand how early life VNC administration modulates the excitability of developing DRG neurons.

Peripheral nerve damage, such as that resulting from mechanical trauma, can evoke downstream changes in nociceptive processing within the spinal superficial dorsal horn (SDH) [13,32,34,49]. It is also becoming increasingly clear that both synaptic plasticity in the SDH network [4,25,31] and the intrinsic membrane excitability of dorsal horn neurons [23,39] can be regulated in a cell type-dependent manner. In addition, as observed in the peripheral nervous system, the effects of nerve damage on the SDH circuit can vary depending on the age at which the insult occurs [47,69], which could partly reflect the ongoing developmental changes in the intrinsic membrane properties and synaptic connectivity of dorsal horn neurons during early life [17,37,38,71]. Unfortunately, in comparison to other forms of peripheral neuropathy, much less is known about how CIPN modulates the functional properties of SDH neurons at any stage of postnatal development.

Therefore, the present study investigated the effects of early life VNC treatment on the intrinsic membrane excitability of adolescent DRG neurons and SDH neurons, including the spinal projection neurons that convey nociceptive signals to the brain and are essential for the generation and maintenance of pathological pain conditions [46,52,61,62]. The results demonstrate that VNC enhances the intrinsic excitability of medium- and large-diameter sensory neurons, as well as lamina I spino-parabrachial neurons, while decreasing the firing of small DRG cells and presumed SDH interneurons. Collectively, these findings suggest

that neonatal VNC administration evokes cell type-specific changes in sensory processing at multiple levels of the developing pain pathway.

2. Methods

2.1. Subjects

Male and female Sprague Dawley rats (n = 46; Harlan, Indianapolis, IN) were used in this study. All animals were housed with their mother prior to weaning on postnatal day (P)21 and were provided food and water ad libitum, and maintained on a 14:10 hour light/dark cycle. All experiments adhered to animal welfare guidelines established by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2. Drug administration

Animals were treated with vincristine as previously described [59]. Vincristine (Tocris, Minneapolis, MN) was dissolved in sterile saline to a concentration of 15 µg/ml and administered intraperitoneally at a dose of 60 µg/kg on P11, 13, 17, 19, and 21 for a total of five injections. Control animals from the same litters received an equivalent volume of sterile saline. This treatment protocol induces mechanical hypersensitivity by P26 which lasts until at least P54 [59].

2.3. Sharp electrode intracellular recordings in isolated whole DRG preparation

Intracellular recordings were obtained from rats at P26–33 during the peak mechanical hypersensitivity (i.e. 5–12 days after the final VNC injection [58]). Animals were anesthetized with isoflurane and a laminectomy was performed at the level of L2–L6. To prevent drying and hypoxia, oxygenated aCSF solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.0 NaH₂PO₄, 1.0 MgCl₂, 2.0 CaCl₂, and 25 glucose (bubbled with 95% O₂ and 5% CO₂, pH = 7.4) was periodically dripped onto the DRG. With the dorsal and ventral roots attached, the L4 or L5 ganglion was then excised and placed in oxygenated aCSF. Under a dissecting microscope, the perineurium and epineurium were carefully peeled away using fine Dumont forceps and the ventral root was separated from the dorsal root and teased off from the DRG. The ganglion was secured by two pieces of fine nylon thread attached to a U-shaped stainless-steel rod in a submersion-type recording chamber (RC-22; Warner Instruments, Hamden, CT) and mounted on the stage of an upright microscope (BX50-WI, Olympus, Center Valley, PA). The DRG was continuously perfused with oxygenated aCSF (see above) at a rate of 2 mL/min at room temperature (22–24°C).

Intracellular recordings were made from sensory neurons near the dorsal surface of the DRG using microelectrodes constructed using single-filament borosilicate glass (1.0 mm outer diameter, 0.5 mm inner diameter; World Precision Instruments, Inc., Sarasota, FL) with a micropipette puller (Sutter Instruments, Novato, CA). Microelectrode impedances were 40–60 MΩ when filled with 3M KCl, 10 mM HEPES, 10 mM EGTA (pH 7.5). Prior to electrode penetration, the focal plane was adjusted to reveal the largest somatic area and a calibrated video image was used to determine cell diameter in two perpendicular directions which were then averaged. DRG neurons were classified based on size as follows: small (< 30 µm), medium (31–49 µm) and large (> 50 µm). Previous studies have shown that classifying DRG

neurons based on soma diameter yields highly similar groups as the use of axonal conduction velocity [22,45,80].

Electrophysiological data were recorded using single-electrode continuous current clamp (AxoClamp-2B, Molecular Devices, Sunnyvale, CA) and analyzed with pClamp 10.7 software (Molecular Devices). Neurons were impaled by applying an oscillating current to the microelectrode once a minimal indent in the membrane was observed and data were acquired after stable recordings were achieved. Membrane resistance (R_m) and membrane capacitance (C_m) were calculated from a hyperpolarization produced in response to a -0.3 nA current injection using an active bridge amplifier. Evoked action potential (AP) parameters were characterized at rheobase, which was determined as the minimum current step required to elicit an action potential (AP) in small-diameter DRG neurons ($-0.2 - 1$ nA delivered in 0.1 nA increments; 75 ms duration) and medium-to-large sensory neurons ($-0.4 - 2$ nA delivered in 0.2 nA increments; 75 ms duration). The AP amplitude was measured as the distance between the peak voltage of the spike and the AP threshold (in mV), and rise time was defined as the corresponding duration in milliseconds. AP half-width (AP50) was calculated as the spike duration at 50% of the peak amplitude. The afterhyperpolarization (AHP) amplitude was calculated as the difference in membrane voltage between the resting potential and the AHP peak, while AHP duration was measured as the time between the AHP peak and the return to the resting membrane potential. Repetitive firing was measured by counting the number of evoked action potentials in response to intracellular current injections (from 0 to 7.0 nA in 0.2 nA increments; 250 ms duration). Instantaneous firing frequency was calculated at each current step as $1/\text{average interspike interval (ISI)}$ and reported at two, four, and six times rheobase values. Cells firing a single AP during the 250 ms depolarization were assigned an instantaneous firing frequency of 4 Hz. The presence of hyperpolarization-activated current (I_h) in DRG neurons was determined by the appearance of a depolarizing “sag” in the membrane voltage during prolonged injections of hyperpolarizing current. Inclusion criteria were a resting membrane potential negative to -40 mV and evoked spikes that overshoot 0 mV.

2.4. Compound action potential recordings in the rat sciatic nerve *in vitro*

At P26–33, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and transcardially perfused with oxygenated ice-cold dissection solution containing the following (in mM): 250 sucrose, 2.5 KCl, 25 NaHCO₃, 1.0 NaH₂PO₄, 6 MgCl₂, 0.5 CaCl₂, and 25 glucose continuously bubbled with 95% O₂/ 5% CO₂. The sciatic nerve was isolated from surrounding tissue, transected distally at mid-thigh level and proximally near the vertebral column (final length: $6 - 12$ mm), then placed in oxygenated aCSF. For compound action potential (CAP) recordings, the sciatic nerve sections were transferred to a submersion-type recording chamber (RC-22; Warner Instruments) and one end was inserted into a suction electrode connected to a constant-current stimulator (Master-8, A.M.P.I., Jerusalem, Israel) while the other end was inserted into a second suction electrode connected to the amplifier headstage. CAPs were recorded at room temperature in response to electrical stimulation ($0 - 1$ mA in 20 μ A increments; 100 μ s duration). A-fiber latencies were measured using the fast component of the observed CAP, consistent with transmission along large myelinated (A α /A β) fibers. C-fiber responses, when visible, were identified as a long-

latency component that followed an A-fiber response, consistent with conduction along unmyelinated fibers. Conduction velocity was calculated as the nerve length divided by the time elapsed from the stimulus onset to the peak of the CAP.

2.5. Retrograde labeling of spino-parabrachial projection neurons

On P3, rat pups (8.0–9.5 g) were anesthetized with a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) and placed in a plaster body mold secured in a stereotaxic apparatus (World Precision Instruments). An incision was made on the scalp, exposing lambda (λ) as well as parts of the parietal and occipital bones. A small hole was created using an OmniDrill 35 (World Precision Instruments) using the following coordinates (in mm, relative to λ): 2.7 caudal, 1.1 lateral, and 4.6 ventral. One injection (150 nl) of FAST DiI oil (2.5 mg/ml; Invitrogen) was delivered to the parabrachial nucleus (PB) using a Hamilton microsyringe (62RN; 2.5 μ l volume) equipped with a 28-gauge needle at a rate of 25 nl/min. After the injection, the needle remained in place for at least one minute to ensure optimal DiI delivery and then was carefully removed from the injection site. The incision was closed with Vetbond (3M; St. Paul, MN) and the pups were placed on a heating pad (37°C) and allowed to recover from anesthesia under close observation prior to being returned to their home cage. Brain tissue was collected following euthanasia and placed in 4% paraformaldehyde at 4°C overnight, and 40–50 μ m coronal sections were cut on a cryostat to ensure accurate DiI injection placement in the parabrachial nucleus.

2.6. *In vitro* spinal cord slice preparation

At P26–33, rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and transcardially perfused with ice-cold dissection solution (see above). After the spinal cord was removed from the vertebral column, the dorsal and ventral roots were cut, and the dura mater was carefully removed. The isolated lumbar enlargement was immersed in low-melting-point agarose (3% in above solution) at 37°C, then placed in an ice mold to cool. Once solidified, the agarose containing the lumbar enlargement was positioned onto a chuck using cyanoacrylate adhesive. Parasagittal slices (250–300 μ m) were cut from the side contralateral to the DiI injection site using a vibrating microtome (7000smz-2; Campden Instruments, Lafayette, IN) and placed in an oxygenated recovery solution containing the following (in mM): 92 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na ascorbate, 2 thiourea, 3 Na pyruvate, 10 MgSO₄, and 0.5 CaCl₂ for 15 min and then placed in an oxygenated aCSF solution (see above) for a further recovery period of 1 hr at room temperature. Following recovery, slices were transferred to a submersion-type recording chamber (RC-22; Warner Instruments), mounted on the stage of an upright microscope (BX51WI; Olympus) equipped with fluorescence to identify DiI-labeled lamina I projection neurons, and perfused with oxygenated aCSF at a rate of 1 – 3 ml/min.

2.7. Patch clamp recordings

Patch clamp electrodes were constructed using thin-walled, single-filament borosilicate glass (1.5 mm outer diameter, 1.12 mm inner diameter; World Precision Instruments, Inc.), with a microelectrode puller (P-97; Sutter Instruments). Current clamp recordings were obtained using patch electrodes filled with an internal recording solution composed of (in mM): 130 K-gluconate, 10 KCl, 10 HEPES, 4 MgATP, 10 Na-phosphocreatine, and 0.3 Na₂-GTP (pH

7.2–7.4, 295–300 mOsm). Pipette resistances ranged from 4 – 6 M Ω and formed seal resistances ≥ 1 G Ω . Dorsal horn neurons with their soma located within 50 μ m of the dorsal white matter were classified as lamina I neurons [44]. Recordings of resting membrane potential (V_{rest}) in DiI-labeled spinal projection neurons and unidentified lamina I neurons were obtained approximately 90 seconds after establishment of the whole cell configuration under current clamp conditions. After adjustment for liquid junction potential, only neurons with a resting potential negative to -50 mV were investigated further. Spontaneous activity and firing patterns were classified at V_{rest} . Membrane resistance (R_m) and capacitance (C_m) were calculated from a hyperpolarization produced in response to a -20 pA current injection from V_{rest} . Evoked action potential properties were characterized at the minimum current intensity (0 to 145 pA in increments of 5 pA; 80 ms duration) required to elicit AP discharge (i.e. rheobase). Repetitive spike discharge was measured via the application of 800 ms intracellular current injections (from -20 to $+170$ pA in 10 pA increments). Instantaneous firing frequency was calculated at each current step as $1/\text{average interspike interval (ISI)}$. Recordings were filtered at 4–6 kHz through a -3 dB, four-pole low-pass Bessel filter, digitally sampled at 20 kHz, and stored on a personal computer (Dell Precision T1650; Dell, Round Rock, TX) using a commercially available data acquisition system (Digidata 1440A with pClamp 10.7 software; Molecular Devices).

2.8. Statistical Analysis

All data were checked for normality using the D'Agostino-Pearson omnibus normality test. Given our prior observation that neonatal VNC evoked mechanical pain hypersensitivity to a similar degree in males and females [59], data from both sexes were pooled for all analyses. Passive membrane and action potential properties were compared between groups using the unpaired Student's t test. For data that did not follow a normal distribution, nonparametric measures were employed (Mann-Whitney U test). Repetitive firing frequency, evoked action potential number, and compound action potential parameters were analyzed with two-way analysis of variance (ANOVA) with Holm-Sidak post hoc tests. Incidence of spontaneous activity, repetitive firing, and hyperpolarization-activated voltage sag (reflecting the presence of I_h) were compared between treatment groups using the Fisher's exact test. The significance level for all tests was set at $P < 0.05$ and all data are reported as mean $\pm$ SEM. All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Prism, La Jolla, CA).

3. Results

To examine the degree to which neonatal vincristine (VNC) treatment influences the excitability of developing sensory neurons, rats received a total of five injections of either vehicle or vincristine (60 μ g/kg; i.p.) between postnatal days (P)11 – 21 and electrophysiological recordings were obtained from dorsal root ganglion (DRG) neurons between P26 and P33, corresponding to the period at which peak mechanical pain hypersensitivity was previously observed [59]. Intracellular microelectrode recordings were obtained from 233 DRG neurons in an intact DRG preparation (see Methods), which included 121 cells from VNC-treated rats and 112 cells from vehicle-treated rats. The VNC treatment group contained 39 large-diameter (> 50 μ m), 58 medium-diameter (31–49 μ m),

and 24 small-diameter ($< 30 \mu\text{m}$) sensory neurons, while 36 large, 54 medium, and 22 small DRG neurons were sampled from the vehicle-treated animals. The experiments investigating the effects of early life VNC on adolescent dorsal horn neurons involved recording from 23 projection neurons and 17 unidentified lamina I neurons in vehicle-treated rats, and 24 projection neurons and 17 unidentified neurons in the VNC-treated group.

3.1. Early life VNC administration increases the excitability of large-diameter primary sensory neurons during adolescence

Representative examples of an evoked action potential and repetitive firing recorded from the soma of a large-diameter DRG neuron are shown in Figures 1A and 1B, respectively. Neonatal VNC treatment failed to alter the minimum current intensity required to initiate an action potential (i.e. rheobase) in large DRG neurons compared to cells from vehicle-treated littermate controls ($P = 0.7817$; Fig. 1C). However, when we examined the ability of large cells to discharge multiple action potentials (APs) in response to longer current injections at two and four times the rheobase levels, 8 of 37 neurons (21.6%) from the VNC group exhibited repetitive firing, whereas 0 of 35 vehicle-treated cells (0%) fired more than one AP ($P = 0.0052$; Fig. 1D). The level of repetitive firing was subsequently measured as both instantaneous firing frequency (see Methods) and the number of APs evoked during an intracellular current injection at 2X and 4X rheobase. VNC significantly increased the instantaneous firing frequency ($F_{(1,70)} = 20.12$, $P < 0.0001$; Fig. 1E), as well as the number of evoked action potentials ($F_{(1,70)} = 9.515$, $P = 0.0029$; Fig. 1F), in the large DRG neurons compared to cells sampled from vehicle-treated littermates. Meanwhile, as summarized in Table 1, the properties of the APs (i.e. AP threshold, amplitude, rise time and half-width) and spike afterhyperpolarizations (AHPs) were similar between experimental groups. Furthermore, early postnatal exposure to VNC did not significantly influence the passive membrane properties (such as V_{rest} , R_m , or C_m), or the prevalence of hyperpolarization-activated voltage sag, in large sensory neurons during adolescence (Table 1).

3.2. Medium-diameter DRG neurons display enhanced intrinsic membrane excitability after neonatal VNC treatment

Unlike large-diameter sensory neurons (Fig. 1C), medium DRG neurons (Fig. 2A, B) exhibited a significantly lower rheobase during adolescence following early life VNC administration compared to vehicle controls ($P = 0.024$; Fig. 2C). Medium-sized cells also demonstrated a greater incidence of repetitive spike discharge as a result of VNC exposure during early postnatal development ($P = 0.034$; Fig. 2D), as was also seen in large sensory neurons. In addition, the rate of instantaneous AP discharge ($F_{(1,179)} = 13.53$, $P = 0.0003$; Fig. 2E), and the total number of evoked spikes ($F_{(1,179)} = 7.668$, $P = 0.0062$; Fig. 2F), was significantly higher in medium sensory neurons from VNC rats compared to those sampled from vehicle-treated controls. In contrast, no significant differences between groups were observed for V_{rest} , R_m , C_m , the incidence of I_h , or the properties of the APs or AHPs in this neuronal population (Table 2). Taken together, these data indicate that the emergence of mechanical pain hypersensitivity during adolescence resulting from neonatal VNC treatment [59] is accompanied by a significant increase in the intrinsic membrane excitability of large- and medium-diameter sensory neurons.

3.3. Early postnatal VNC administration reduces the excitability of small DRG neurons during adolescence

In small primary afferent neurons (Fig. 3A, B), VNC treatment during the neonatal period did not significantly alter rheobase compared to vehicle-injected controls at P26–33 ($P = 0.112$; Fig. 3C). In addition, the incidence of repetitive firing did not significantly differ between treatment groups ($P = 0.1038$; Fig. 3D). However, unlike the observed effects in large- and medium-sized DRG cells, small neurons displayed a significant reduction in instantaneous firing frequency following early life VNC administration in comparison to the vehicle-treated group ($F_{(1,83)} = 5.214$, $P = 0.025$; Fig. 3E). The mean number of action potentials evoked during the prolonged current injection was also significantly reduced in small sensory neurons from VNC-treated rats compared to controls ($F_{(1,83)} = 6.71$, $P = 0.0113$; Fig. 3F). In addition, the amplitude ($P = 0.0136$) and duration ($P = 0.0229$) of the AHP in this population decreased following early life VNC, while no changes were observed in the passive membrane properties, incidence of I_h , or the parameters of the evoked action potentials (Table 3).

3.4. Early life VNC exposure does not alter the conduction velocities of adolescent A- or C-fibers in vitro

Abnormal sensory nerve conduction velocities (CV) have been reported in clinical and experimental studies of chemotherapy-induced peripheral neuropathy across multiple ages [14,21,26,43,51,55]. Therefore, using in vitro recordings of A- and C-fiber compound action potentials (CAPs) from the sciatic nerve (Fig. 4A), we sought to determine whether our model of pediatric vincristine-induced neuropathic pain was associated with impaired CV at the onset of peak mechanical pain hypersensitivity during adolescence. The data demonstrate that VNC treatment during early life fails to significantly alter the CVs of A-fibers in comparison to the vehicle-treated group at either P27 or P34 ($F_{(1,27)} = 0.0151$, $P = 0.9031$, $n = 7 - 10$ in each group; Fig. 4B). Similar results were observed when analyzing the CAPs corresponding to small-diameter unmyelinated fibers, as no significant differences were noted in the CVs of C-fiber evoked responses between animals treated with vincristine during the neonatal period compared to the vehicle group at either time point ($F_{(1,12)} = 0.3352$, $P = 0.5733$, $n = 3 - 5$; Fig. 4C). Collectively, these data suggest that the alterations in the function of adolescent DRG neurons evoked by neonatal VNC does not include impaired conduction of sensory signals along the peripheral nerve.

3.5. Early life VNC treatment enhances the intrinsic excitability of adolescent lamina I projection neurons

Spinal projection neurons were identified via retrograde transport of DiI delivered to the parabrachial nucleus (PB) at P3 (see Methods). At P26–33, sagittal spinal cord slices were prepared and patch clamp recordings were obtained from identified spino-PB neurons located in lamina I. As shown in Figure 5A, exposure to vincristine during early life produced a marked reduction in rheobase levels within adolescent lamina I projection neurons ($P = 0.0218$; $n = 22 - 23$). However, there were no significant differences in the overall prevalence of spontaneous action potential discharge ($P = 0.7666$; Fig 5B) or the mean rate of spontaneous firing (Table 4) between VNC- and vehicle-treated groups at this

time point. In response to intracellular current injections of longer duration (Fig. 5C), lamina I projection neurons from VNC-treated animals exhibited significantly higher instantaneous firing frequencies across a range of stimulus intensities compared to littermate controls ($F_{(1,579)} = 23.66$, $P < 0.0001$; Fig. 5D). Finally, we observed no other significant differences between groups in regards to the passive membrane properties or AP properties of spino-PB neurons (Table 4).

3.6. Unidentified lamina I neurons exhibit decreased repetitive firing following neonatal VNC administration

Given that the intrinsic membrane properties of spinal SDH neurons are developmentally regulated in a cell type-specific manner [37,38,71], we also examined the effects of early life VNC treatment on the intrinsic excitability of unidentified lamina I neurons at the same ages. Since projection neurons only account for ~5% of all lamina I neurons, the remaining ~95% of neurons correspond to propriospinal and local circuit interneurons [3,65] and therefore likely represent the population of unidentified neurons sampled in this study. Neonatal VNC exposure did not significantly impact rheobase ($P = 0.9482$, $n = 17$ in each group; Fig. 6A) or the incidence of spontaneous activity ($P = 0.4646$; Fig. 6B) in this neuronal population at P26–33. Interestingly, we did observe a slight but significant decrease in the average instantaneous firing frequency in unidentified lamina I neurons from animals injected with VNC during early life compared to vehicle-treated controls ($F_{(1,425)} = 6.82$, $P = 0.0093$; Fig. 6C, D). Nonetheless, we failed to observe any significant changes in passive membrane properties or in most properties of the evoked APs (i.e. spike threshold, amplitude, or half-width) in adolescent lamina I neurons following neonatal VNC treatment (Table 5). Overall, these results point to cell type-dependent changes in the intrinsic membrane properties of developing SDH neurons in the aftermath of pediatric VNC administration.

4. Discussion

VNC administration in rats between postnatal days (P)11 and 21 evokes mechanical hyperalgesia and allodynia by P26 [59]. The major goal of this study was to investigate VNC-induced changes in the intrinsic membrane properties of first- and second-order neurons in the ascending pain pathway during the period when pain hypersensitivity first emerges. Collectively, the results demonstrate that neonatal VNC treatment alters neuronal excitability in a cell type-dependent manner in both the DRG and spinal superficial dorsal horn (SDH) during adolescence.

Following early life exposure to VNC, large- and medium-diameter sensory neurons exhibited a significantly greater ability to fire repetitively (Fig. 1, 2), which is interesting given that the selective block of A β primary afferents alleviates spontaneous pain following chemotherapy [76]. The observed hyperexcitability could reflect direct actions of VNC on sensory neurons, as the acute application of VNC to the distal axons of primary afferents in culture can reportedly produce membrane depolarization at the soma [56]. Overall, our results are consistent with prior studies of adult DRG neurons under conditions of CIPN, which showed that A-fibers exhibit a lower rheobase and greater discharge in response to

graded electrical stimuli [64], while other passive and active membrane properties were preserved [79]. One notable exception is that we failed to witness spontaneous firing in adolescent DRG neurons of any size after neonatal VNC administration, while abnormal spontaneous discharge in sensory neurons appears to be a hallmark feature of CIPN in adults [74,75,79]. However, the degree to which this apparent discrepancy reflects true age-dependent differences in spontaneous activity following chemotherapy, as opposed to differences in experimental design (such as the choice of recording temperature [35]) requires further investigation. In addition, while VNC has been shown to reduce the conduction velocity (CV) of both A- and C-fibers within sensory nerves [64,73,75], we observed no significant differences between the VNC- and vehicle-treated littermates when measuring CV using in vitro compound action potential recordings. Interestingly, clinical reports during and after treatment of childhood ALL have shown that many patients experience clinical symptoms of peripheral neuropathy despite normal nerve conduction properties [21,26,43,55].

The molecular mechanisms driving the VNC-evoked hyperexcitability of sensory neurons during adolescence remain to be determined. It will certainly be important to better understand the neuro-immune interactions occurring within the peripheral nerves in the aftermath of early life VNC treatment. For example, the infiltration of the sciatic nerve by circulating monocytes, and their activation by the chemokine CX3CL1, is instrumental to the generation of neuropathic pain in adult mice treated with VNC [48,53]. Unfortunately, the influence of age on the immune response occurring within peripheral nerves following the administration of chemotherapeutic agents remains poorly understood. Meanwhile, recent work has clearly demonstrated that adolescence represents a critical time period during which the spinal cord immune response to peripheral nerve damage switches from anti-inflammatory to pro-inflammatory in nature [47]. It also remains unclear whether the level of mitochondrial dysfunction and oxidative stress produced in sensory neurons following chemotherapy, which are thought to play a prominent role in the sensitization of nociceptive pathways [1,15,53,75], depends on the age at which the treatment occurs.

C-fibers from adult rats with established VNC-induced peripheral neuropathy become hyper-responsive to electrical and mechanical stimulation [63,64]. Furthermore, exposure to chemotherapy can enhance the expression of transient receptor potential (TRP) channels such as TRPA1 and TRPV1 [12,20,81], as well as Na_v1.7 channels [41], in mature sensory neurons. However, adolescent small-diameter sensory neurons exhibited a significant reduction in repetitive action potential discharge following neonatal VNC treatment (Fig. 3). While our recent work showed that early life VNC failed to modulate the reflex withdrawal thresholds in response to noxious heat or cold [59], more sensitive assays of thermosensation [8,50] could be employed to detect more subtle VNC-evoked changes in the ability to discriminate between different temperatures. Indeed, pediatric patients with vincristine-induced neuropathic pain tend to experience deficits in thermal discrimination when warm or cool objects are applied to the skin [33], as opposed to the cold allodynia or elevated noxious heat detection thresholds seen in adults [14].

Nonetheless, we cannot exclude the possibility that examining later time points after VNC administration would reveal hyperexcitability within small DRG neurons, as human studies

suggest that the symptoms of CIPN are initially attributed to large, myelinated sensory neurons and then progress to small myelinated, and eventually unmyelinated, nociceptors [14]. Given that the die-back of intraepidermal nerve fibers (IENFs) following neonatal chemotherapy occurs with a delay relative to the onset of mechanical hypersensitivity [58], while no such delay is observed in adults [2,6,60], it is possible that the small-diameter sensory neurons are in the initial stages of dysfunction during the adolescent period and could eventually transition into a state of hyperexcitability. It should also be noted that the present studies employed a single dosing protocol consisting of five individual injections of vincristine (60 µg/kg). It is possible that cumulative doses exceeding 300 µg/kg would increase membrane excitability in small DRG neurons, as the symptoms of vincristine-induced peripheral neuropathy are positively correlated with the total dose and duration of treatment [14,19,33,54]. Finally, the design of the current experiments would not allow for the detection of enhanced TRP channel function, or an increased sensitivity to inflammatory mediators, in small DRG neurons after early life chemotherapy.

It is well known that mechanical damage to the peripheral nerve can lead to downstream alterations in nociceptive processing within the spinal superficial dorsal horn [13,32,34,49]. Functional plasticity in spinal sensory encoding has also been observed in adult rats with chemotherapy-induced neuropathic pain, where elevated spontaneous activity, greater after-discharges to noxious mechanical stimuli, and abnormal wind-up in wide dynamic range (WDR) neurons within the deep dorsal horn have been reported after vincristine [73], paclitaxel [9], or cisplatin administration [10]. A multitude of mechanisms potentially contribute to this enhanced firing of dorsal horn neurons under neuropathic conditions, including a reduction in glutamate clearance [9,72], activation of sphingolipid signaling [27], and an upregulated expression of the $\alpha_2\delta_1$ subunit of voltage-gated calcium channels [77]. Our current results suggest that CIPN is also accompanied by cell type-dependent modifications in the intrinsic membrane properties of spinal neurons residing in the superficial laminae, as early life VNC treatment enhanced the intrinsic excitability of lamina I spino-parabrachial neurons while slightly decreasing repetitive firing in adjacent, unidentified neurons (95% of which will correspond to propriospinal or local circuit interneurons [3]). Given that lamina I projection neurons represent a major conduit for ascending nociceptive transmission to the brain [65] and are essential for the generation of neuropathic and inflammatory pain [46,52], an enhanced ability of this population to fire repetitively after neonatal VNC administration (Fig. 5) is predicted to directly increase the signaling gain of the spinal pain circuit [40] and thereby contribute to the mechanical hypersensitivity which emerges during adolescence [59].

One notable limitation is that the presumed interneurons sampled in the present studies were not identified as excitatory or inhibitory, although glutamatergic neurons clearly predominate in the rat SDH [58,66]. Therefore, it will ultimately be important to elucidate the degree to which early life chemotherapy differentially regulates the intrinsic membrane properties of GABAergic vs. glutamatergic interneurons in the developing SDH, as well as identify the effects of neonatal VNC on synaptic transmission within the network. Previous studies have illustrated that chemotherapeutic agents can suppress synaptic inhibition in the adult dorsal horn via multiple mechanisms including altered intracellular Cl^- homeostasis [11] and a miRNA-mediated reduction in the expression of the GABA synthetic enzyme

GAD67 [24]. Furthermore, CIPN has been associated with the generation of reactive oxygen species (ROS) in the dorsal horn [15,30], which is known to suppress spinal GABA release under neuropathic conditions [78]. Interestingly, ROS have also been shown to modulate synaptic plasticity in a cell type-dependent manner in the SDH, by mediating LTP at afferent synapses onto spinal projection neurons while simultaneously driving LTD at sensory synapses onto GABAergic interneurons [4]. However, since the consequences of peripheral tissue and nerve injury for synaptic function in the SDH clearly depend on the age at which the injury occurs [36], future studies are needed to confirm that neonatal VNC treatment also compromises synaptic inhibition in the developing dorsal horn. If so, this would be predicted to unmask low-threshold A-fiber synaptic input to lamina I projection neurons [67]. As a result, the documented increase in the excitability of medium and large DRG neurons after neonatal VNC could potentially combine with a weaker inhibitory tone within the SDH to potentiate low-threshold primary afferent drive to ascending projection neurons, and thereby contribute to mechanical pain hypersensitivity following early life chemotherapy.

In conclusion, the above results demonstrate that pediatric exposure to vincristine modulates the intrinsic membrane excitability of adolescent neurons at multiple sites along the ascending pain pathway. Given the prolonged mechanical pain hypersensitivity evoked by early life vincristine [59], it will clearly be important to delineate the complete time course of these changes and to elucidate the underlying ionic mechanisms.

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Figure 1. Vincristine (VNC) treatment during early life enhances the excitability of large-diameter DRG neurons during adolescence.

(A) Representative trace of an evoked action potential (AP) from a large-diameter DRG neuron at rheobase. Scale bars: 10 mV, 2 ms. (B) Example of a large-diameter neuron from vehicle-treated (upper) or VNC-treated (lower) rats demonstrating the firing of multiple APs in response to suprathreshold stimulation. Scale bars: 20 mV, 20 ms. (C) VNC did not impact rheobase levels ($P = 0.7817$, $n = 36 - 39$, Mann-Whitney test). (D) VNC significantly increases the incidence of repetitive firing (RP) in large-diameter cells, as this trait was not observed in any cells from vehicle (VEH)-treated animals ($**P = 0.0052$, $n = 36 - 39$, Fisher's exact test). (E) Plot of instantaneous firing frequency in large sensory neurons at a stimulus intensity of 2X and 4X rheobase, illustrating a significant increase in firing rate after early life VNC exposure compared to vehicle-treated controls ($F_{(1,70)} = 20.12$, $P < 0.0001$, $n = 26$ for each group, two-way ANOVA). (F) Graph of the number of evoked action potentials at 2X and 4X rheobase, illustrating a significant increase in AP discharge in VNC cells compared to control cells which consistently fired a single AP ($F_{(1,70)} = 9.515$, $P = 0.0029$, $n = 26$ in each group, two-way ANOVA). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; Holm-Sidak post hoc analysis. ANOVA, analysis of variance.

Figure 2. Medium-diameter sensory neurons become hyperexcitable after early life VNC. (A) Representative trace of an evoked action potential (AP) in a medium-diameter DRG neuron. Scale bars: 10 mV, 5 ms. (B) Example of a medium-diameter cell from vehicle-treated (upper) or VNC-treated (lower) rats showing the discharge of multiple APs in response to suprathreshold stimulation. Scale bars: 20 mV, 20 ms. (C) VNC administration significantly reduced rheobase in medium-diameter cells compared to controls ($*P = 0.0241$, $n = 54 - 58$, unpaired t -test). (D) Medium-diameter sensory neurons display an increased prevalence of firing multiple APs in response to sustained depolarizing pulses after VNC treatment compared to controls ($*P = 0.034$, Fisher's exact test). (E) Plot of instantaneous firing frequency as a function of 2X, 4X, and 6X rheobase, demonstrating that VNC significantly accelerates the rate of spike discharge with respect to vehicle (VEH)-treated controls ($F_{(1,179)} = 13.53$, $P = 0.0003$, $n = 43 - 47$ for each group, two-way ANOVA). (F) Neonatal VNC also increased the number of generated action potentials during sustained current injection ($F_{(1,179)} = 7.668$, $P = 0.0062$, $n = 43 - 47$, two-way ANOVA). ** $P < 0.01$; *** $P < 0.001$; Holm-Sidak post hoc analysis.

Figure 3. Pediatric vincristine exposure decreases repetitive firing in small-diameter DRG neurons during adolescence.

(A, B) Representative traces showing an evoked AP (A) and evoked spike train (B) from a small-diameter DRG neuron in the vehicle (upper) and VNC (lower) groups. Scale bars: 20 mV, 20 ms. (C) Rheobase values in this population were not significantly affected by neonatal VNC administration ($P = 0.112$, $n = 21 - 23$ for each group, Mann-Whitney test). (D) The incidence of repetitive firing ability did not significantly differ between treatment groups ($P = 0.1038$, $n = 21 - 23$, Fisher's exact test). (E) Plot of instantaneous firing frequency at 2X, 4X, and 6X rheobase illustrating a VNC-induced attenuation of repetitive firing rate ($F_{(1,83)} = 5.214$, $P = 0.025$, $n = 16 - 22$, two-way ANOVA). (F) In small sensory neurons from vehicle (VEH)-treated animals, the number of evoked APs increased with greater stimulus intensities, whereas the firing of neurons from the VNC-treated group remained similar across the range of stimulation intensities examined ($F_{(1,83)} = 6.71$, $P = 0.0113$, $n = 16 - 22$, two-way ANOVA; $*P < 0.05$).

Figure 4. VNC did not significantly impact the conduction velocities of primary afferent fibers at P27 or P34.

(A) Representative example of a compound action potential (CAP) recorded from the adolescent rat sciatic nerve in vitro. Scale bars: 2 mV, 5 ms. (B, C) A-fiber (B) and C-fiber (C) conduction velocities during adolescence were unaffected following early life exposure to VNC. A-fibers: ($F_{(1,27)} = 0.0151$, $P = 0.9031$, $n = 7 - 10$ in each group, two-way ANOVA). C-fibers: ($F_{(1,12)} = 0.3352$, $P = 0.5733$, $n = 3 - 5$, two-way ANOVA).

Figure 5. Enhanced intrinsic excitability in adolescent lamina I spino-parabrachial neurons following neonatal VNC exposure.

(A) Rheobase was significantly reduced in ascending spinal projection neurons from adolescent rats treated with vincristine during early life compared to vehicle controls ($*P = 0.0218$, $n = 22 - 23$, Mann-Whitney test). (B) VNC did not impact the overall rate of spontaneous action potential (AP) generation in projection neurons relative to controls ($P = 0.7666$, $n = 22 - 23$, Fisher's exact test). (C) Representative traces of repetitive action potential discharge in projection neurons from vehicle-(upper) and vincristine-treated (lower) animals. Scale bars: 15 mV, 50 ms (upper); 20 mV, 50 ms (lower). (D) Plot of instantaneous firing frequency as a function of stimulus intensity revealing an enhanced responsiveness of projection neurons to suprathreshold depolarizing current injections following neonatal vincristine treatment ($F_{(1, 579)} = 23.66$, $****P < 0.0001$, $n = 22 - 23$, two-way ANOVA).

Figure 6. Early postnatal vincristine exposure significantly reduces the rate of action potential discharge in unidentified lamina I neurons.

(A) Vincristine administration during the neonatal period did not significantly impact rheobase in unidentified lamina I neurons during adolescence ($P = 0.9482$, $n = 17$ in each group, unpaired t-test). (B) There was no significant effect of neonatal VNC on the prevalence of spontaneous firing ($P = 0.4646$, $n = 17$, Fisher's exact test). (C) Representative traces of repetitive action potential (AP) discharge in unidentified lamina I neurons from adolescent rats following exposure to vehicle (upper) and vincristine (lower) during early life. Scale bars: 20 mV, 50 ms. (D) Plot of instantaneous firing frequency as a function of stimulus intensity illustrating a significantly lower rate of evoked AP discharge in unlabeled lamina I neurons following VNC administration compared to littermate controls treated with vehicle ($F_{(1,425)} = 6.82$, $P = 0.0093$, $n = 17$, two-way ANOVA; $**P < 0.01$).

Table 1.

Effects of vincristine on the electrophysiological properties of large-diameter DRG neurons during adolescence

Large Diameter ($\geq 50 \mu\text{m}$)	Vehicle	Vincristine
V_{rest} (mV)	-62.77 ± 1.61	-61.61 ± 1.57
R_{m} (M Ω)	16.19 ± 1.53	16.35 ± 1.16
C_{m} (pF)	71.99 ± 5.22	83.67 ± 8.4
No. of cells with I_{h}	13/36	22/36
AP Threshold (mV)	-29.29 ± 5.52	-33.9 ± 2.74
AP Amplitude (mV)	64.66 ± 2.14	66.27 ± 1.96
AP Rise Time (ms)	0.52 ± 0.02	0.54 ± 0.03
AP Half-width (ms)	0.45 ± 0.03	0.45 ± 0.03
AHP Amplitude (mV)	10.25 ± 0.9	10.25 ± 0.97
AHP Duration (ms)	2.63 ± 0.29	2.52 ± 0.28

Data are expressed as mean $\pm$ SEM. V_{rest} = resting membrane potential; R_{m} = membrane resistance; C_{m} = capacitance; AP = action potential; AHP = afterhyperpolarization.

Table 2.

Effects of vincristine on the electrophysiological properties of medium-diameter DRG neurons

Medium Diameter (31–49 μm)	Vehicle	Vincristine
V_{rest} (mV)	-60.48 ± 1.19	-57.44 ± 1.32
R_{m} (M Ω)	24.92 ± 1.79	23.34 ± 1.44
C_{m} (pF)	64.65 ± 3.71	61.45 ± 3.34
No. of cells with I_{h}	11/54	17/58
AP Threshold (mV)	-34.08 ± 2.32	-33.43 ± 2.17
AP Amplitude (mV)	62.92 ± 1.75	59.48 ± 1.92
AP Rise Time (ms)	0.77 ± 0.05	0.74 ± 0.05
AP Half-width (ms)	0.86 ± 0.1	0.67 ± 0.07
AHP Amplitude (mV)	10.14 ± 0.89	11.87 ± 0.69
AHP Duration (ms)	3.97 ± 0.5	3.26 ± 0.21

Data are expressed as mean $\pm$ SEM. V_{rest} = resting membrane potential; R_{m} = membrane resistance; C_{m} = capacitance; AP = action potential; AHP = afterhyperpolarization.

Table 3.

Effects of vincristine on the electrophysiological properties of small-diameter DRG neurons

Small Diameter ($\leq 30 \mu\text{m}$)	Vehicle	Vincristine
V_{rest} (mV)	-53.72 ± 1.39	-56.23 ± 1.81
R_{m} (M Ω)	32.18 ± 2.18	37.33 ± 2.42
C_{m} (pF)	41.47 ± 2.51	47.7 ± 2.43
No. of cells with I_{h}	3/22	2/24
AP Threshold (mV)	-23.86 ± 1.79	-23.97 ± 1.78
AP Amplitude (mV)	47.05 ± 2.56	50.8 ± 1.99
AP Rise Time (ms)	1.46 ± 0.09	1.4 ± 0.09
AP Half-width (ms)	1.86 ± 0.16	1.97 ± 0.14
AHP Amplitude (mV)	12.87 ± 1.2	$9.03 \pm 1.03^{\#}$
AHP Duration (ms)	5.74 ± 0.54	$4.25 \pm 0.3^*$

Data are expressed as mean $\pm$ SEM.* $P < 0.05$, unpaired t -test;# $P < 0.05$, Mann-Whitney test. V_{rest} = resting membrane potential; R_{m} = membrane resistance; C_{m} = capacitance; AP = action potential; AHP = afterhyperpolarization.

Table 4.

Effects of vincristine on the electrophysiological properties of identified lamina I projection neurons

Lamina I Projection Neurons	Vehicle	Vincristine
V_{rest} (mV)	-66.55 ± 2.58	-65.76 ± 2.05
R_{m} (M Ω)	574.1 ± 44.15	702.7 ± 65.54
C_{m} (pF)	69.13 ± 3.24	76.28 ± 4.95
Spontaneous Activity (Hz)	1.34 ± 0.51	1.33 ± 0.4
AP Threshold (mV)	-48.5 ± 1.24	-50.9 ± 1.73
AP Amplitude (mV)	68.88 ± 3.93	70.64 ± 4.19
AP Rise Time (ms)	2.39 ± 0.28	2.0 ± 0.25
AP Half-width (ms)	1.58 ± 0.08	1.37 ± 0.09

Data are expressed as mean $\pm$ SEM. V_{rest} = resting membrane potential; R_{m} = membrane resistance; C_{m} = capacitance; AP = action potential.

Table 5.

Effects of vincristine on the electrophysiological properties of unidentified lamina I neurons during adolescence

Unidentified Lamina I neurons	Vehicle	Vincristine
V_{rest} (mV)	-69.35 ± 2.98	-66.5 ± 3.00
R_{m} (M Ω)	953.5 ± 64.46	847.3 ± 87.52
C_{m} (pF)	57.91 ± 5.71	65.87 ± 5.9
Spontaneous Activity (Hz)	1.40 ± 0.7	0.62 ± 0.38
AP Threshold (mV)	-33.15 ± 1.61	-29.96 ± 1.7
AP Amplitude (mV)	59.14 ± 4.79	55.09 ± 5.3
AP Rise Time (ms)	1.5 ± 0.19	$2.01 \pm 0.28^*$
AP Half-width (ms)	0.97 ± 0.08	1.03 ± 0.12

Data are expressed as mean $\pm$ SEM. [#] $P < 0.05$, Mann-Whitney test. V_{rest} = resting membrane potential; R_{m} = membrane resistance; C_{m} = capacitance; AP = action potential.