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Neuropathic pain after chronic nerve constriction may not correlate with chloride dysregulation in mouse trigeminal nucleus caudalis neurons

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Introduction

Chronic pain afflicts over 100 million Americans, and costs the USA up to \$650 billion/year in medical treatment and lost productivity.⁸ Because the underlying pathophysiology of chronic pain is largely unknown, most chronic pain patients are highly resistant to current pharmaceutical or surgical therapies.

One of the mechanisms proposed for the pathophysiology of chronic, neuropathic pain involves reduced inhibition in the spinal cord, resulting in amplified activity of spinal neurons that relay nociceptive information to CNS structures (reviewed by).^{36, 43} In their seminal study, Coull et al⁹ showed that this reduced spinal inhibition can result from reduced expression of the potassium-chloride exporter, KCC2, and a consequent increase in chloride in nociceptive dorsal horn neurons. This disruption in the transmembrane chloride gradient depolarizes the equilibrium potential of currents generated by GABA_A receptors (E_{GABAA}), reducing the inhibitory effects of GABA responses, and, in a small number of spinal neurons, renders the GABA responses paradoxically excitatory.^{9, 36} Subsequent studies provided further support for this chloride dysregulation hypothesis, and implicated also changes in the chloride importer, NKCC1, as well as in microglia-derived growth factors that regulate chloride transporters.^{18, 29, 36, 37}

We reasoned that if chloride dysregulation is causally related to disinhibition and to increased pain perception, it should persist as long as increased signs of pain are present. Alternatively, chloride dysregulation may be involved in the initial phase of chronic pain, which, as we have shown, is mechanistically distinct from the late, maintenance phase.³⁵ Specifically, we showed that the *induction* of hyperalgesia depends on inputs from the injured nerve, whereas the *maintenance* of hyperalgesia requires central mechanisms involving descending serotonergic drive. There are currently conflicting reports, some

indicating that chloride dysregulation is transient,^{33, 48, 50} and others arguing that it is long-lasting.^{20, 34}

To test these predictions we took advantage of a neuropathic model of chronic pain, in which the infraorbital branch of the trigeminal nerve is loosely ligated.^{3, 35} We computed E_{GABAA} from perforated-patch, *in vitro* recordings of neurons in the spinal trigeminal nucleus caudalis (SpVc), and used quantitative, real-time polymerase chain reaction (qRT-PCR) to determine the expression of the chloride transporters, KCC2 and NKCC1. In all experiments we studied these variables both at the early and late phases of chronic pain.

Methods

We adhered to accepted standards for rigorous study design and reporting to maximize the reproducibility and translational potential of our findings as described in Landis et al²³ and in ARRIVE (Animal Research: Reporting In Vivo Experiments) Guidelines. Animals were randomly allocated to experimental or control groups, as described in Kim and Shin.²² In all experiments the investigators were blinded to animal condition. A coded key of all specimens evaluated was kept and not shared with the investigators performing the experiments until data analyses were completed. We performed a power analysis to estimate the requires samples needed for each experiment.

Animals

All animal protocols were approved by the University of Maryland's Institutional Animal Care and Use Committee, and adhered to National Research Council guidelines.³² To identify GABAergic neurons during *in vitro* recordings we used transgenic mice that express green fluorescent protein (GFP) under the control of the GAD2 promoter; these mice were developed and characterized by Szabo and collaborators.²⁶

We used both male and female mice for all experiments. Factorial analysis of variance revealed no significant interaction of sex with any measured variable, we combined data from both sexes.

CCI-ION

We adapted, to the mouse, a rat model of neuropathic pain, evoked by chronic constriction of the infraorbital nerve (CCI-ION).^{3, 35} Animals were anesthetized with ketamine and xylazine, and intra-oral surgery was performed using aseptic conditions. An incision was made along the gingivobuccal margin, beginning after the first molar. The ION was freed from surrounding connective tissue, and loosely tied using silk thread (4-0), 1 to 2mm from the nerve's exit at the infraorbital foramen. We used silk thread, rather than chromic gut as originally described by Benoist et al³, because silk ligatures demonstrate more stable neuropathic pain behaviors in mouse CCI-ION models.⁴⁴ In sham-operated mice the nerve was exposed without ligature.

Behavior

A series of calibrated von Frey filaments were applied to the orofacial skin, at the cutaneous site innervated by the ION. An active withdrawal of the head from the probing filament was defined as a response. We used the up-down method to determine withdrawal thresholds, as described previously.⁷

Electrophysiological recordings

Horizontal slices (300 μ m thick) containing SpVc were prepared from adult (40 day old) mice, following the method described by Ting et al.⁴² For recordings, slices were continually perfused (2 ml/min) with artificial cerebrospinal fluid (ACSF) containing, in mM: NaCl (119), KCl (2.5), NaH₂PO₄ (1.2), NaHCO₃ (2.4), glucose (12.5), MgSO₄•7H₂O (2), CaCl₂•2H₂O (2). To obtain perforated patch clamp recordings, the pipette tip was filled with a solution containing (in mM) 135 potassium gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 2 MgCl₂ (pH 7.4). The pipette was back-filled with this same solution supplemented with 2 μ g/ml gramicidin D. Recordings were performed when access resistance was stable between 20 to 50 M Ω . Changes in access resistance greater than 20% were considered evidence that the cell membrane was ruptured, leading to termination of the recording. Recordings were performed in voltage clamp mode with a holding potential of -70 mV.

RNA extraction and quantitative RT-PCR

Mouse SpVc tissue punches were collected at intervals of 3 to 5 days or 12 to 15 days following sham or CCI-ION surgery, and from age-matched naïve controls (5 animals per group) and stored at -80°C. RNA was extracted using Trizol (Invitrogen) and the MicroElute Total RNA Kit (Omega) with a DNase step (Qiagen). All RNA quantities were measured on a Nanodrop. 500 ng RNA was taken from each sample for cDNA synthesis by reverse transcriptase, using iScript cDNA synthesis kit (Bio-Rad). 5 ng cDNA was loaded from each sample for quantitative polymerase chain reaction (qPCR) with PerfeCTa SYBR Green FastMix (Quanta). Each reaction was run in duplicate and quantification of mRNA changes was performed using the $\Delta\Delta$ CT method described previously,^{6, 25} using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. We used the following primers – KCC2 forward sequence: AAGGGCAGAGAGTACGATGG; KCC2 reverse sequence: CCTGGGGTAGGTTGGTGTAG; NKCC1 forward sequence: ACTTCAGTGCCCAGTCAAAG; NKCC1 reverse sequence: AATGGGGCAACTTTCCATGTG; GAPDH forward sequence: AGGTCGGTGTGAACGGATTTG; GAPDH reverse sequence: TGTAGACCATGTAGTTGAGGTCA. Primers were validated using a standard serial dilution of template cDNA. We performed no-template controls as well as no reverse transcriptase; all controls were negative.

Results

Behavior

To test if chronic constriction injury of the infraorbital nerve (CCI-ION) resulted in behavioral signs of neuropathic pain, we measured withdrawal thresholds to mechanical stimuli—calibrated von Frey filaments—applied to the buccal pad.

Twenty two animals were tested before, and 3 to 5 days after CCI; paired t-test showed a significant reduction in thresholds after CCI ($p < 10^{-4}$; Fig. 1A), from a mean of 8.4 g (7.7-9.1 g, 95% confidence interval), to a mean of 2.5 g (1.1 to 0.2 g, mean $\pm$ CI). Similarly, 15 animals tested before (9.2 g, 8.8 to 9.6 g, mean $\pm$ CI), and again 12 to 14 days after CCI (1.6 g, 1.3 to 1.9 g, mean $\pm$ CI) also showed a significant reduction in withdrawal thresholds (paired t-test, $p < 10^{-4}$; Fig. 1A). These findings indicate that CCI-ION results in mechanical hyperalgesia that lasts at least 14 days, as previously reported by us and others.^{35, 45}

Electrophysiology

To test the hypothesis that neuropathic pain is associated with the appearance of depolarizing, excitatory GABA inputs onto SpVc neurons, we obtained perforated-patch, voltage clamp recordings from lamina I & II neurons in SpVc slices. We used 17 mice 12 to 14 days after CCI-ION, 7 animals 3 to 5 days after CCI-ION, and 21 sham-operated mice. Sham-operated mice were tested at 3 to 5 days ($n=9$ mice) or 12 to 14 days after sham surgery. Because the results from these two sham groups were statistically indistinguishable, we combined these sham results.

We recorded steady state currents at a range of voltage commands (-120 to +10 mV) applied to the recorded neurons. Figure 2A shows representative current traces recorded, from the same neuron, in the presence of TTX and muscimol (GABA_A agonist). To compute whole cell currents evoked by activation of GABA_A receptors, we subtracted from the muscimol & TTX traces the traces obtained in the presence of TTX alone. The resulting difference trace represents currents mediated specifically by GABA_A receptors (Fig. 2A). To estimate the reversal potential of these currents (E_{GABAA}) we measured the steady state current at each holding potential, and constructed current-voltage (IV) plots, as shown in Figure 2B. For each neuron, we estimated E_{GABAA} from these plots, and averaged these reversal potentials for neurons recorded from each experimental group.

There was no significant difference in reversal potential recorded from control (naïve) and sham-operated mice ($p=0.5$; Fig. 3A); Therefore, in all subsequent comparisons we used data from the sham group as controls. ANOVA revealed significant differences ($p < 10^{-4}$) between sham, CCI-ION 3 to 5 days and CCI-ION 12 to 14 days. In SpVc neurons recorded from sham-operated mice, mean chloride reversal potential was -70.3 mV (-71.25 to -69.25 mV, 95% CI, $n=16$). At 3 to 5 days after CCI-ION there was a small (~7%), yet significant ($p < 10^{-4}$; *posthoc* Tukey's multiple comparisons test) increase in E_{GABAA} to -65.4 (-67.97 to -62.9 mV CI, $n=16$). However, this change was transient, because at 12 to 14 days after CCI-ION E_{GABAA} (-70.4 mV; -71.06 to -69.82 mV, $n=18$), was not statistically distinguishable from that in sham-operated mice ($p=1.0$; *posthoc* Tukey's multiple comparisons test).

The use of transgenic mice allowed us to target some of our recordings to GABAergic interneurons labeled with green fluorescent protein transgenically expressed under the GAD2 promoter. ANOVA revealed no significant differences ($p=0.054$) between sham, CCI-ION 3 to 5 days and CCI-ION 12 to 14 days (Fig. 3B). Thus, E_{GABAA} of GAD-GFP neurons did not change significantly from sham-operated levels (-70.1 mV, -71.7 to -68.6 mV 95% confidence interval, $n=8$) at 3 to 5 days (-66.9 mV, -71.6 to -62.2 mV, $n=7$) or at 12 to 14 days after CCI-ION (-70.7 mV, -71.7 to -69.6 mV, $n=9$) after CCI ION.

In summary, CCI-ION was associated with a transient and small (5 mV difference; 8%) reduction in E_{GABAA} in SpVc neurons, but not in GABAergic neurons. E_{GABAA} values returned to pre-CCI levels by 12 days after CCI-ION.

As recently explicated by Prescott, De Koninck and collaborators,¹³ estimation of chloride reversal potentials is complicated by several potential issues (see also Discussion). One is that if a neuron is tested while experiencing only a weak chloride load, KCC2 will have excess chloride extrusion capacity, such that changes in $E_{chloride}$ will only become evident when KCC2 reductions are substantial (see also).¹² Thus, although our measurements of E_{GABAA} were sensitive enough to detect ~8% changes at 5 days after CCI, they may have not been sensitive to detect smaller changes at 14 days.

To assess this possibility we computed, for each neuron, the slope of the IV curve (Fig. 2B), considering the slope a proxy for direct measurement of GABA_A conductance (although a change in slope might also reflect a changes in driving force).¹³ Figure 3C plots the average and 95% confidence intervals slopes computed for neurons from each of three groups. Note that the confidence intervals of the sham and 5 day CCI groups are separate almost throughout the voltage range, whereas the slope of the 14 day CCI group overlaps with the two others. We quantify this by comparing the slopes of these curves (Fig. 3D). Kruskal-Wallis test, with Dunn's correction for multiple comparisons, reveals that slopes computed for sham vs 5 day CCI are significantly different ($p=0.003$), whereas sham vs 14 day CCI ($p=0.09$) and 5 day CCI vs 14 day CCI ($p>0.9$) are not.

The significant difference in slopes support the conclusion that chloride flux is significantly reduced 5 days after CCI, and may return to control levels after 14 days. These findings further support the interpretation that E_{GABAA} changes only transiently after CCI-ION.

PCR

Neuronal intracellular chloride concentrations are regulated, in part, by chloride transporters.^{5, 19, 29} Therefore, we used qRT-PCR to compare mRNA of two neuronal transporters, NKCC1 and KCC2. We used 15 mice for each of the following groups: naïve, 3 to 5 days post-CCI, 12-14 days post-CCI, 3 to 5 days post sham operation, and 12 to 14 days post sham operation. We averaged, for each animal, data from duplicate PCR samples for each primer.

As depicted in Figure 4, there were no significant differences in mRNA levels of either NKCC1 ($p=0.15$, Kruskal-Wallis analysis of variance) or KCC2 ($p=0.69$). The data (Fig. 4) suggest that, at 3 to 5 days after CCI, there was a slight decrease in KCC2; therefore, we

compared the values obtained at 3 to 5 days from CCI and sham-operated mice, but found no significant difference ($p>0.08$; Mann-Whitney). Thus, these data suggest that CCI-ION does not result in significance differences in the expression of mRNA for NKCC1 or KCC2.

Discussion

CCI-ION results in only transient changes in E_{GABAA}

We found that CCI-ION results in a transient and modest depolarization of the GABA_A reversal potential (E_{GABAA}). This change was restricted to the first 5 days after nerve injury, and E_{GABAA} reverted to normal values during the second week post CCI-ION. This transient change did not occur in GABA neurons (Fig. 3D). In preliminary experiments we found that this transient change occurred in SpVc neurons that project to the parabrachial nucleus (identified with fluorescent beads retrogradely labeled from the parabrachial). Because the small sample size ($n=3$ neurons recorded 3 to 5 days after CCI) was insufficient for statistical analysis, we did not present these preliminary data. Importantly, at 12 to 14 days after CCI ($n=5$) we found no significant changes in E_{GABAA} , compared to controls ($n=5$; Mann-Whitney $p=0.2$).

Because of the transient nature of the small change in E_{GABAA} , and because CCI-ION produces hyperalgesia that lasts many weeks (Fig. 1),^{35, 45} we conclude that persistent CCI-induced hyperalgesia in mice may not be substantially influenced by transient changes in E_{GABAA} and in intracellular chloride concentrations.

A caveat in interpreting data on currents recorded at neuronal somata is that such recordings report on currents in the peri-somatic region. Because of dendritic cable properties such recordings may fail to detect currents in more distal dendritic segments.³⁹ As a result, we cannot exclude the possibility that our recordings failed to detect larger changes in E_{GABAA} due to currents generated in distal dendrites,⁴¹ or due to differences in chloride between somatic and dendritic compartments.¹ The relatively low access resistance of perforated patch recordings mitigates this concern. Also mitigating the concern are findings that neurons in the medullary dorsal horn form GABAergic synapses on their somata and proximal dendrites,⁴⁶ suggesting that space clamp limitations were modest.

We recognize that reliable estimates of anion reversal potential require that the recorded neuron experience a large enough chloride load.¹³ Our ability to detect changes as small as 8% in E_{GABAA} and in IV slopes at 5 days post-CCI diminish this concern. Nevertheless, we cannot exclude the possibility that our analyses were insensitive to detect changes $<8\%$ at 14 days after CCI. Indeed, in a computational study Doyon et al¹² suggest that inconspicuous changes in chloride fluxes, and mild KCC2 hypo-function, might be sufficient to dysregulate coding in neuronal networks. It remains to be determined how such small changes in chloride fluxes might affect SpVc networks.

Here we tested the effects of CCI on chloride fluxes mediated by GABA_A. We did not test the role of glycinergic transmission, which plays an important role in regulating SpVc and spinal dorsal horn activity, and in several chronic pain conditions.^{16, 36, 37, 43} However, because glycine acts through an ionotropic receptor that produces its effects through chloride

current, and because our findings suggest that CCI-Pain is not associated with persistent changes in chloride fluxes or in KCC2, it is unlikely that dysregulation of chloride transmission plays a key role in our model of chronic pain.

Of note, Prescott et al,³⁶ in a computational study, have shown that small changes in E_{GABA} , like the transient changes we report here (from about -70 to -65 mV) are likely to have no, or only minimal effects on the activity of dorsal horn circuits (see also).⁹

CCI and chloride transporters

The hypothesis that CCI-Pain is associated with persistent changes in chloride equilibrium potential is also incompatible with our finding that CCI-ION was not associated with changes in the expression of either of the chloride transporters studied. Thus, there was neither a decrease in KCC2, the “mature” transporter that extrudes chloride from neurons, nor an increase in the “immature” transporter, NKCC1, which imports chloride.^{2, 29, 38} That the transient increase in E_{GABA} at 3 to 5 days after CCI-ION was not associated with similar changes in the expression of chloride transporters might reflect an inability of qRT-PCR to detect very small changes in gene expression. It is also possible that CCI-ION is associated with other changes to these transporters, such as changes in protein expression, their trafficking, surface density, phosphorylation state, and oligomerization.^{4, 15, 17, 21, 47} Such mechanisms may, potentially, lead to reduced activity through KCC2 or increased activity through NKCC1, leading to increased intracellular chloride concentrations. This, in turn, could lead to E_{GABA} becoming depolarizing and excitatory. However, our data suggest that changes in E_{GABA} are small and transient, and, therefore, are likely to have a modest role in the early phases of neuropathic pain, but may not contribute significantly to the later, maintenance phase of chronic pain.

Nerve transection was reported to result in down-regulation of KCC2 that lasts at least 21 days.³⁴ Neuropathic pain in diabetic rats is associated with a decrease in KCC2, lasting at least 8 weeks.²⁰ There are conflicting reports regarding pain after spinal cord injury, with Hasbargen et al¹⁸ reporting transient changes in chloride transporters, whereas others^{10, 28} reporting long-lasting changes in their expression and in E_{GABA} ; However, the latter two studies are based on statistical comparisons that fail to account for repeated measures, rendering these results uninterpretable.

Although we cannot exclude the possibility that these contradictory reports reflect methodological or species differences, the present findings are consistent with previous studies, and their conclusion that chronic, neuropathic pain may result in transient, but not permanent changes in E_{GABA} .

Role of E_{GABA} in transient pain

Neuropathic pain, including that induced by nerve constriction, is often described as occurring in phases, with an early phase lasting, in rodents, about a week, and a later, permanent phase.^{45, 49} We and others have shown that these phases are dissociable.³⁵ The transient changes in E_{GABA} reported here may have a role in the pathogenesis of the early phase of neuropathic pain. Indeed, our findings are consistent with an *in situ* hybridization and immunocytochemistry report that CCI-ION results in only transient changes in mRNA

and protein levels of chloride transporters.⁴⁸ These findings are consistent also with previous reports of transient changes in chloride transporters after formalin or CFA injections.^{33, 50} Similarly, ligation of the sciatic nerve is associated with a transient (less than a week) decreased expression of KCC2.³⁰ Also relevant is that inflammation of the tooth pulp causes down-regulation of KCC2 and up-regulation of NKCC1 in SpVc, and that these changes last only two days.²⁴

Nevertheless, other studies suggest that some of these changes may be longer-lasting. For example, in their seminal study, Coull et al⁹ appear to have recorded changes in E_{GABA} that lasted more than two weeks after peripheral nerve injury (although this is not explicitly stated in the manuscript). An important distinction between the present results and the Coull et al findings (and later findings from that group) is that we bath-applied muscimol, whereas the other group locally and transiently applied GABA_A agonists. Although this methodological difference may affect the recorded kinetics, we note that Lu et al²⁸ also bath applied GABA, and detected changes in E_{GABA} .

Role of GABA in chronic pain

Maladaptive changes in GABA function may be causally responsible for chronic pain in several forms, including reduced GABA and GAD levels,^{14, 31, 40} increased functional expression of GABA transporters,¹¹ reduced GABA release,⁵¹ and decreased excitatory drive to GABAergic neurons.²⁷ Thus, that changes in chloride dynamics may have only a transient, or minor role in the pathophysiology of chronic pain does not mitigate the pivotal role that maladaptive plasticity in GABAergic transmission plays in chronic, neuropathic pain, as reviewed elsewhere.^{36, 43}

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Figure 1. CCI-ION produces persistent hyperalgesia. Data represent means and 95% confidence intervals of withdrawal thresholds to mechanical stimuli applied to the face. p values obtained from ANOVA followed by Tukey's multiple comparisons test.

Figure 2.

Estimation of EGABAA from perforated path recordings of SpVc neurons. A: Current evoked in response different voltage commands (500 msec duration). Current traces obtained, from the same neuron, in the presence TTX (top), and after addition of muscimol (middle). Lower trace is depicts the difference between the to top traces. B: EGABAA estimated from the steady state phase of current traces depicted in A. C: Digitized image showing neurons identified in the slice preparation as GABAergic (GAD-GFP).

Figure 3.

CCI-IION is associated with a transient reduction in EGABAA. A: Group averages and 95% confidence intervals for EGABAA estimated for each of the groups of neurons analyzed. B: Group averages and 95% confidence intervals of IV curves computed for GABAergic neurons. C: Means and 95% confidence intervals for IV curves were computed for each of the animal groups. D: Group averages and 95% confidence intervals depicting the slopes of IV curves computed for each of the animal groups. Open circles show data from individual neurons. p values obtained from ANOVA followed by Tukey's multiple comparisons test.

Figure 4.

CCI-ION is not associated with significant differences in mRNA levels for the chloride transporters, NKCC1 or KCC2. Shown are group averages and 95% confidence intervals of mRNA levels measured with qRT-PCR.