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# Migraine and peripheral pain models show differential alterations in neuronal complexity

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## Abstract

**Objective:** Our laboratory has recently shown that there is a decrease in neuronal complexity in head pain processing regions in mouse models of chronic migraine-associated pain and aura. Importantly, restoration of this neuronal complexity corresponds with anti-migraine effects of known and experimental pharmacotherapies. The objective of the current study was to expand this work and examine other brain regions involved with pain or emotional processing. We also investigated the generalizability of our findings by analyzing neuronal cytoarchitectural changes in a model of complex regional pain syndrome (CRPS), a peripheral pain disorder.

**Methods:** We used the nitroglycerin (NTG) model of chronic migraine-associated pain in which mice receive 10 mg/kg NTG every other day for 9 days. Cortical spreading depression (CSD), a physiological corelate of migraine aura, was evoked in anesthetized mice using KCl. CRPS was induced by tibial fracture followed by casting. Neuronal cytoarchitecture was visualized with Golgi stain and analyzed with Simple Neurite Tracer.

**Results:** In the NTG model, we previously showed decreased neuronal complexity in the trigeminal nucleus caudalis (TNC) and periaqueductal gray (PAG). In contrast, we found increased neuronal complexity in the thalamus and no change in the amygdala or caudate putamen in this study. Following CSD, we observed decreased neuronal complexity in the PAG, in line with decreases in the somatosensory cortex and TNC reported with this model previously. In the CRPS model there was decreased neuronal complexity in the hippocampus, as reported by others; increased complexity in the PAG; and no change within the somatosensory cortex.

**Conclusions:** Collectively these results demonstrate that alterations in neuronal complexity are a feature of both chronic migraine and chronic CRPS. However, each type of pain presents a unique cytoarchitectural signature, which may provide insight on how these pain states differentially transition from acute to chronic conditions.

KEYWORDS
aura, migraine, mouse, neuroplasticity, cytoskeleton

**Abbreviations:** CRPS, complex regional pain syndrome; CSD, cortical spreading depression; NTG, nitroglycerin; OIS, optical intrinsic imaging; PAG, periaqueductal gray; SCx, somatosensory cortex; TNC, trigeminal nucleus caudalis; VEH, vehicle; vlPAG, ventrolateral periaqueductal gray; VPM, ventral posteromedial nucleus of the thalamus.

## INTRODUCTION

Chronic pain syndromes, including chronic migraine, are debilitating conditions that affect approximately 20% of American adults, greatly impacting their quality of life.[1] Despite its wide prevalence, the pathophysiology that drives the transition of pain from an acute state to a chronic one is still not entirely known.[2] One possible mechanism implicated in many neuropsychiatric conditions, including chronic pain, is alterations in neuroplasticity.[3] Within the broad range of neuroplasticity, recent evidence has suggested the importance of cytoarchitectural alterations in the regulation of many psychiatric conditions.[4,5] A key component of the cytoarchitecture is microtubules, which are in a constant state of dynamic instability.[6] Microtubules facilitate cellular responses to injury and regulate many neuronal functions, including neurite branching, axonal transport, and signaling.[7,8] Importantly, alterations in neurite structure, including gross changes in neuronal complexity and dendritic spine density, are observed in animal models of neuropathic pain disorders.[9,10] Therefore, it is possible that alterations in neuronal cytoarchitecture may be fundamental for initiating and/or maintaining pain chronicity.

Chronic pain can encompass disorders effecting both central and peripheral processes.[11] An especially common centrally regulated pain disorder is chronic migraine. Chronic migraine affects up to 2% of the general population and is defined as having at least 15 headache days a month for a minimum of 3 months.[12,13] While much has been recently learned about the pathophysiology responsible for the transition of migraine from an acute to chronic state the underlying mechanisms are still being explored.[14] A chronic migraine model, which uses the human migraine trigger nitroglycerin (NTG), as well as a model of cortical spreading depression (CSD), were recently shown to result in alterations in cytoarchitecture within key migraine regulating regions.[15] This, along with human research showing alterations in gray matter volume following chronic migraine further indicates a possible causal mechanism between headache chronicity and alterations in cytoarchitectural dynamics.[16,17] Other chronic pain disorders have also been shown to have alterations in neuronal complexity in humans and animal models, including complex regional pain syndrome (CRPS).[18–21] CRPS originally presents as an acute/peripheral disorder, but over time can transition to a chronic/centrally regulated pain disorder.[22] The acute phase is characterized by warmth of the affected skin, while in the chronic phase there is no peripheral limb warmth or inflammation, but there is chronic pain in humans and allodynia in animal models as well as debilitating cognitive deficits.[23,24] As with chronic migraine much is still unknown about the transition from acute to chronic CRPS.[2] Gaining a better understanding of the changes in the cytoarchitecture following chronic pain disorders could allow for the development of novel therapeutics that are affective in multiple pain conditions.

The aim of this study was to further investigate alterations that occur following chronic migraine and assess if similar alterations in cytoarchitecture are observed in a hind paw model of CRPS. We previously showed that there were neuronal cytoarchitectural differences in key migraine pain processing regions following the NTG model of chronic migraine.[15] These results revealed a novel mechanism responsible for the transition of migraine from an episodic to a chronic condition. We sought to build on these original findings by exploring other brain regions related to headache processing and emotional and affective aspects of pain.[25,26] In the NTG model of chronic migraine we examined neuronal complexity in the thalamus, central amygdala, and the caudate putamen. We further expanded on neuroplasticity induced by CSD and examined the periaqueductal gray (PAG). Finally, we compared our cytoarchitectural results with another chronic pain model, CRPS. We hypothesized that different pain states would result in unique cytoarchitectural changes.

## MATERIALS AND METHODS

### Animals

Experiments were performed on adult C57BL6/J mice (Jackson Laboratories) aged 9–16 weeks. The chronic migraine experiments used both male and female mice, while the CSD work used only females. All experiments performed with the CRPS model used only male mice. All mice weighed between 20 and 30 g for the length of the study. To ensure health, weight was recorded on each day for all experiments. Mice were group housed in a 12–12 h light–dark cycle, in which the lights were turned on at 7:00 a.m. and then turned off at 7:00 p.m. Both food and water were available ad libitum. All experiments were conducted in a blinded fashion. The procedures for all studies were approved by the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety Committee, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International guidelines and the Animal Care Policies of the University of Illinois at Chicago. The results are reported according to Animal Research: Reporting of In Vivo Experiments. The CRPS experiments were done in collaboration with Dr. Vivianne Tawfik at Stanford University. The in vivo experiments modeling CRPS were done at Stanford University and in accordance of their animal care committees. No animals were seen to have adverse effects and all animals were included in the statistical analysis. Euthanasia was consistent with American Veterinary Medical Association guidelines. Mice were euthanized with $CO_2$ followed by decapitation.

### Chronic migraine sensory sensitivity testing

Different groups of animals were used for each experiment. The mice for the chronic migraine experiment were counter-balanced on the first day based upon their original basal threshold. Mice were tested in a behavior room, which was completely separated from the vivarium. The testing room had low light and low noise conditions. All tests were performed between 8:00 a.m. and 4:00 p.m. For cephalic testing, animals were habituated to the testing rack for 2 days prior

to the original test day. The rack also contained 4 oz paper cups that the mice habituated to. On subsequent test days mice were placed on the rack with the cups 20 min prior to that day's basal measurement. The up and down method was used to assess punctate mechanical stimuli.[27] Manual von Frey hair filaments with a bending force between 0.008 and 2 g were used in these experiments. A response in the cephalic measures was defined as repeated shaking, pawing at the face, or cowering from the filament following stimulation. The first filament used was 0.4 g and following no response a heavier filament (up) was used. Alternatively, if there was a response a lighter filament (down) was used next. This up and down pattern was repeated for four filaments following the initial response.

## Complex regional pain syndrome sensitivity testing

In vivo experiments and testing were conducted by Dr. Vivianne Tawfik's laboratory at Stanford University. Logarithmically increasing sets of von Frey filaments were used in a range of 0.007 to 6.0 g. The filaments were applied to the plantar hind paw until a bend occurred. A positive response was a withdrawal from the filament within 4 s. The up and down method was also used for these experiments and a 50% withdrawal mechanical threshold were calculated for the mice.

## Nitroglycerin model of chronic migraine

Nitroglycerin was purchased at a concentration of 5 mg/ml, in 30% alcohol, 30% propylene glycol, and water (American Reagent). For the chronic NTG experiment, the NTG was diluted prior to testing on each day with 0.9% saline to a concentration of 1 mg/ml and a dose of 10 mg/kg. The 0.9% saline served as the vehicle (VEH) control. Mice were injected with NTG ip every other day for 9 days. On days 1, 5, and 9 mice were tested before and 2 h following NTG injection.

## Cortical spreading depression model

The CSD model used in this study is based on previous work by Ayata[28,29] that is commonly used to screen potential migraine therapeutics. For these studies only female mice were used and mice were randomly assigned to sham or CSD groups. The skulls of the mice were thinned to form a cortical window. For the surgery the mice were anesthetized with isoflurane induction 3%–4%; maintenance 0.75% to 1.25%; in 67% $N_2$/33% $O_2$). Once adequate anesthetic level was assessed the mice were placed on a stereotaxic frame on a homoeothermic-heating pad. Life signs were monitored throughout the experiment including core temperature, non-peripheral oxygen saturation, heart rate, and respiratory rate (PhysioSuite; Kent Scientific Corporation). To ensure proper anesthetic depth mice were frequently tested for tail and hind paw reactivity.

CSD events were verified using optical intrinsic imaging (OIS) and electrophysiological recordings as previously described.[30] Following anesthesia the skin was cleared from the skull and a rectangular region of ~2.5 × 3.3 $mm^2$ (~0.5 mm from sagittal, and ~1.4 from coronal and lambdoid sutures) of the right parietal bone was thinned to transparency with a dental drill (Fine Science Tools). Following successful window creation mineral oil was applied to the surface to improve transparency for video recording. A green light-emitting diode (530 nm) was further used to illuminate the skull to aid in recording (1-UP; LEDSupply). Cortical surface reflectance detected by OIS was collected with a lens (HR Plan Apo 0.5 × WD 136) through a 515LP emission filter on a Nikon SMZ 1500 stereomicroscope (Nikon Instruments). Images were acquired at 1–5 Hz using a high-sensitivity Universal Serial Bus monochrome charge-coupled device (CCE-B013-U; Mightex) with 4.65-micron square pixels and 1392 × 1040 pixel resolution.

Lateral to the window two burr holes were drilled. These burr holes were drilled deeper than the thinned window such that exposure to the dura was achieved, but not so deep as to damage the dura. Local field potentials were recorded using an electrode filled with saline that was inserted into the dorsal burr hole and subsequently attached to an amplifier. Placing a silver wire beneath the skin grounded the animals. Basal electrophysiological measurements were measured for 1 h prior to KCl application to induce CSD. KCl (1M) was dripped into the rostral burr hole at a rate that ensured a continual pool, but not so much that excess spilled over onto the thinned skull. The pool of KCl was maintained for 1 h of recording. Sham mice had the skull thinned and the burr holes drilled, but they did not receive KCl drip or have an electrode placed as punctate stimuli can produce a CSD itself. Following CSD recording mice were euthanized and brains were collected for Golgi staining.

## Complex regional pain syndrome model

Dr. Tawfik's lab performed the CRPS model. Mice were anesthetized using isoflurane and had a closed right distal tibial fracture followed by casting.[31] The right hind limb was wrapped in gauze and a hemostat was used to fracture the distal tibia. The hind limb was wrapped using casting tape from the metatarsals to the spica formed around the abdomen. The cast was applied only to the plantar surface and a window was left to prevent constriction. Twenty-one days following casting the casts were removed. Mice had a basal measure prior to fracture, one 21 days after fracture (acute phase), and then at 7 weeks (chronic phase). Following completion of the seventh week the mice were sacrificed and their brains underwent the Golgi staining technique. Afterward they were shipped to the University of Illinois at Chicago for tissue processing and analysis.

## Golgi staining

All Golgi staining was done using the FD Rapid Golgi Stain Kit (FD NeuroTechnologies, Inc.). Chronic NTG mice were sacrificed on day 10 following anesthesia using isoflurane and then decapitated. CSD/

sham mice were similarly sacrificed following the hour of CSD recordings. CRPS animals were sacrificed 7 weeks after casting and were subsequently used in the same Golgi staining behavior. Brains were removed and then rinsed in distilled water. An impregnation solution of A and B was prepared in advance and brains were placed in this solution for 1 week in the dark. Following this they were placed into solution C for 72 h. Brains were then flash frozen in 2-mehtylbutane and cut on a cryostat to 100 μm slices. The slices were then put on slides and stained using the kit procedure.

### Neurite tracing

After tissue processing all images were taken at 20× magnification and a *Z*-stack was created through different focal planes. The FIJI program Simple Neurite Tracer was used to process the neurons.[32] The software was also used to count the number of branch points, overall length, and Sholl analysis. Sholl analysis used the soma as the center point and 20 pixels as the consecutive circles.

### Neuron selection

The tracers were blinded to which group the images belonged. Six to eight relatively isolated neurons were randomly chosen per mouse. The neurons were fully impregnated with Golgi stain. An atlas and clear anatomical markers were used to take images from the region of interest. Somatosensory cortex neurons were taken from layer IV of the primary somatosensory barrel cortex. For NTG and CSD paradigms we intended to have at least $n = 5–7$/group with six neurons traced/mouse/region. For CRPS we intended four mice/group with eight neurons traced/mouse/region. These numbers were based on our previous study.[33] Because we investigated changes at the cellular level, an individual neuron represented a single sample.

### Statistical analysis

Appropriate sample size was chosen for each experiment based on previous literature and the following power analysis: minimal detectable difference in means = 0.3, expected standard deviation of residuals = 0.15, desired power = 0.8, alpha = 0.05, $n = 6$/group. Data analysis was performed using GraphPad Prism version 8.00 (GraphPad). Two-tailed *t*-tests were used to compare neurite complexity between groups. The level of significance for all tests was set to $p < 0.05$. Histograms were used to verify assumptions. Two-way repeated measures analysis of variance was used to analyze allodynia data with factors of treatment (NTG/VEH) and time. Post hoc analyses were conducted using Holm-Sidak post hoc test. Post hoc analysis was only conducted when *F* values achieved significance of $p < 0.05$. Grubbs' test was used to identify outliers, and none were found. All mice and data points were included in the analysis. All values in text and in figures are reported as mean ± standard error of the mean.

## RESULTS

### Chronic NTG treatment produces cytoarchitectural changes in pain circuits

We used a previously established model of chronic migraine by treating male and female C57BL6/J mice every other day for 9 days with NTG or VEH (Figure 1A).[34] We measured periorbital response to mechanical stimulation on days 1, 5, and 9 (Figure 1A). NTG produced a significant decrease in basal cephalic mechanical thresholds, and mice were sacrificed on day 10, 24 h after the final treatment (Figure 1B). Brains were Golgi stained to investigate possible neuronal cytoarchitectural alterations. We examined the ventral posteromedial nucleus of the thalamus (VPM) as it has been implicated as a key player in regulating allodynia and sensitization from the trigeminal circuit (Figure 1C).[35–38] Examination of the number of branch points per neuron revealed an increase in the total number of branches in the NTG treated mice (Figure 1D, 23% increase relative to VEH). There was no significant increase in the overall length of neurons (Figure 1E) or interactions as assessed through Sholl analysis (Figure 1F,G). While there was not a significant change in all measures, the increase of branching of neurons within the VPM is an interesting contrast to the previous findings of decreased branching and complexity in the trigeminal nucleus caudalis (TNC), ventrolateral periaqueductal gray (vlPAG), and the somatosensory cortex (SCx) following chronic NTG.[15]

### Chronic NTG treatment did not induce changes in neuronal complexity in central amygdala or caudate putamen

Migraine results in a host of symptoms outside of pain, including alterations in emotional regulation and cognitive dysfunction.[39,40] The amygdala and the basal ganglia have both been implicated in migraine pathophysiology.[41,42] We investigated if there were any changes within the central amygdala following chronic NTG treatment (Figure 2A). There was no significant change in the number of branch points (Figure 2B), total neuronal length (Figure 2C), or in the complexity of the neurons assessed through Sholl analysis (Figure 2D,E). Similar results were observed for the caudate putamen (Figure 2F). There was no significant difference in branches (Figure 2G), total neuronal length (Figure 2H), or complexity (Figure 2I,J). These data show that the alterations in cytoarchitecture following chronic NTG, while widespread, do not affect every region.

### Cortical spreading depression results in neuroplasticity in the periaqueductal gray

CSD is an electrophysiological phenomenon considered to be the physiological correlate of migraine aura.[43] CSD is mechanistically distinct from the NTG model of migraine pain, and migraine preventatives have been shown to decrease CSD events.[29]

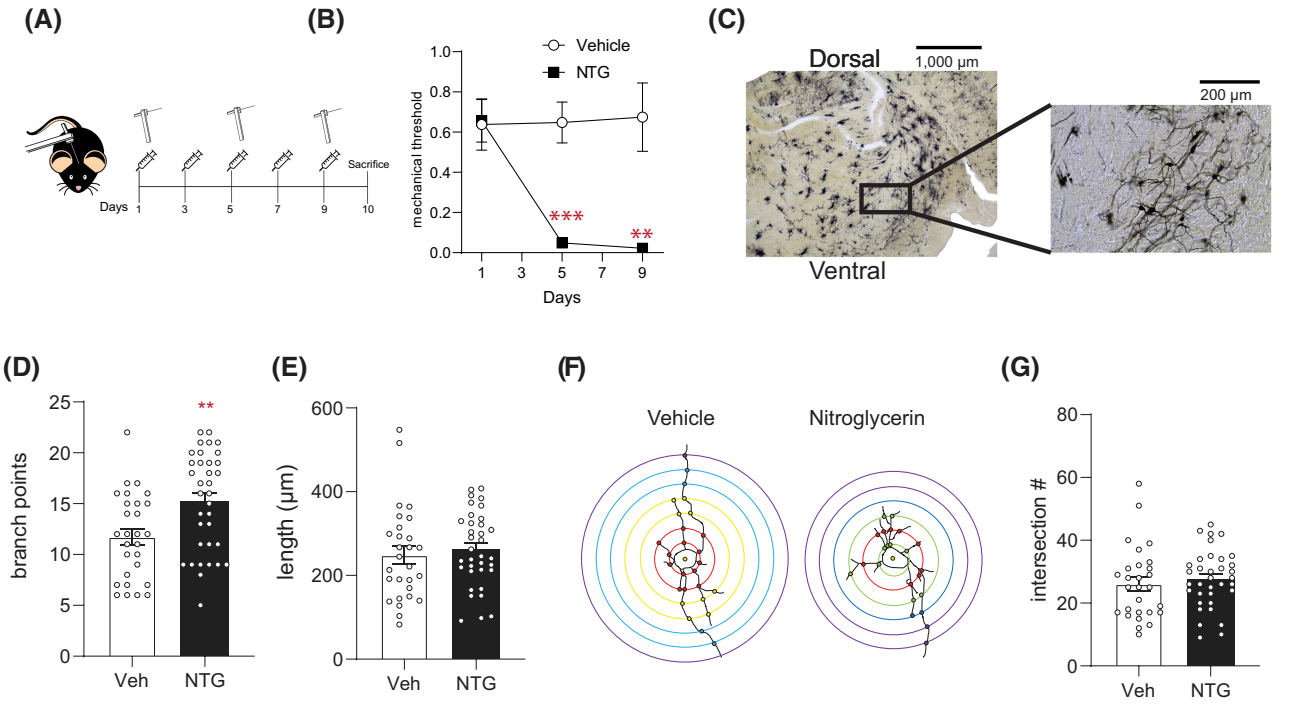


**FIGURE 1** Chronic nitroglycerin (NTG) treatment increased neuronal complexity within the ventral posteromedial nucleus of the thalamus. A, Schematic of testing and injections, M&F C57BL6/J mice were treated with chronic intermittent NTG (10 mg/kg, ip) or vehicle (VEH) for 9 days and on day 10 tissue was collected for Golgi staining. B, Periorbital mechanical thresholds were accessed prior to VEH/NTG administration on days 1, 5, and 9. NTG produced severe cephalic allodynia, $p < 0.001$ effect of drug, time, and interaction, two-way repeated measures analysis of variance and Holm-Sidak post hoc analysis. ***$p < 0.001$, **$p < 0.01$ relative to vehicle on same day $n = 5–6$/group. C, Representative image taken of the Golgi stained ventral posteromedial nucleus of the thalamus at 4× (left) and 20× (right). D, The number of branch points per neuron were significantly increased following treatment with chronic NTG. Unpaired *t*-test **$p < 0.01$ E, Total neuron length was also measured but showed no significant change. Unpaired *t*-test. F, Representative Sholl image of neuron from VEH (left) and NTG (right) treated mice. G, Sholl analysis showed no significant changes between the NTG and VEH-treated groups. Unpaired *t*-test. $n = 5–6$ mice per group, 6 neurons per mouse.

We examined mice that underwent multiple KCl stimulated CSDs or sham procedures (Figure 3A,B). Following the thinned skull procedure, continual KCl was dripped onto the dura for 1 h. Sham mice were used as controls and underwent the same skull thinning procedure, were anesthetized for the same duration of time, but did not receive KCl infusion. Following the CSD/sham procedure, brains were collected and underwent Golgi staining and the vlPAG was examined (Figure 3C). Neurons within the vlPAG were found to have a significantly decreased number of branch points following repeated CSD events (Figure 3C, 18% decrease relative to sham). Neurons in this region also showed decreased length (Figure 3D, 18% decrease relative to sham). We did not observe a significant change in interactions as determined by Sholl analysis (Figure 3E–G). These data show that a primarily cortical driven phenomenon can have widespread impact on brain regions involved with pain processing.

## Complex regional pain syndrome resulted in varying alterations in neuronal complexity depending on the brain region examined

Given our findings that widespread alterations in cytoarchitecture are present in two distinct models of migraine, we determined if these changes could be observed in a peripheral chronic pain state. To do this we used an established model of CRPS.[31,44] Briefly, mice underwent a distal tibial fracture followed by 3 weeks of casting. As casting and fracture on their own can cause CRPS, uninjured mice were used as control. Following removal of the cast at 3 weeks, animals had sustained basal hypersensitivity that persisted through 20 weeks post fracture.[31] These mice were sacrificed at week 7, which is considered well within the chronic/central phase, and had their tissue processed with Golgi staining. While the pain is the primary symptom associated with CRPS, cognitive impairment and memory deficits are also common affecting upward of 50% of chronic CRPS patients.[45,46] Previously another lab using a similar model showed alterations in hippocampal complexity. We first replicated these results and examined cytoarchitectural alterations in the dorsal hippocampus in CRPS and control mice (Figure 4A). Investigation of branching revealed a significant decrease in the total number of branches following CRPS (Figure 4B, 24% decrease relative to control). Changes in branches also correlated with decreased neuronal length (Figure 4C, 21% decrease relative to control), and interactions as determined by Sholl analysis (Figure 4D,E, 24% decrease relative to control).

This study, and previous work,[15] showed decreased neuronal complexity within the vlPAG following chronic migraine and CSD.

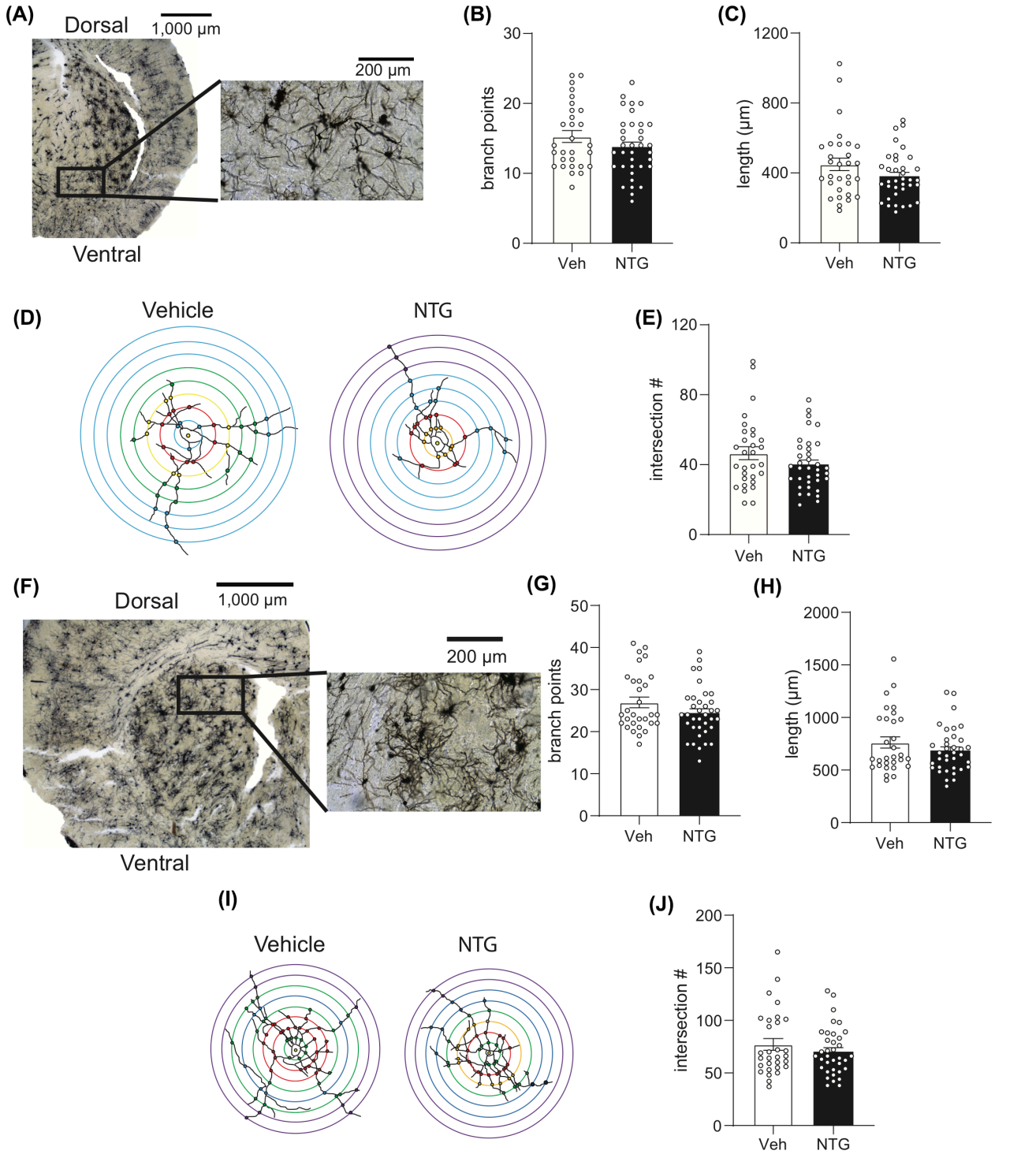


**FIGURE 2** Chronic nitroglycerin (NTG) did not alter neuronal complexity in the amygdala or caudate putamen. Mice were treated with chronic intermittent NTG or vehicle (VEH) as described above. A, Representative image of central amygdala 4× (left) and 20× (right). B, Neurons from this region were analyzed for number of branch points and showed no significant change. Unpaired *t*-test. C, Total neuron length was also found to show no significant change. Unpaired *t*-test. D, Representative Sholl analysis image of neurons from mouse treated with VEH (left) and NTG (right). E, Sholl analyses were conducted and there was no significant change in the number of intersections within the central amygdala. Unpaired *t*-test. F, Representative image of the caudate putamen 4× (left) and 20× (right). G, Neurons from this region were analyzed for number of branch points and showed no significant change. Unpaired *t*-test. H, Total neuron length also showed no significant change. Unpaired *t*-test. I, Sholl analysis image from mouse treated with VEH (left) and NTG (right). J, No significant change in the number of intersections within the caudate putamen. Unpaired *t*-test. $n = 5–6$/mice/group, 6 neurons per mouse.

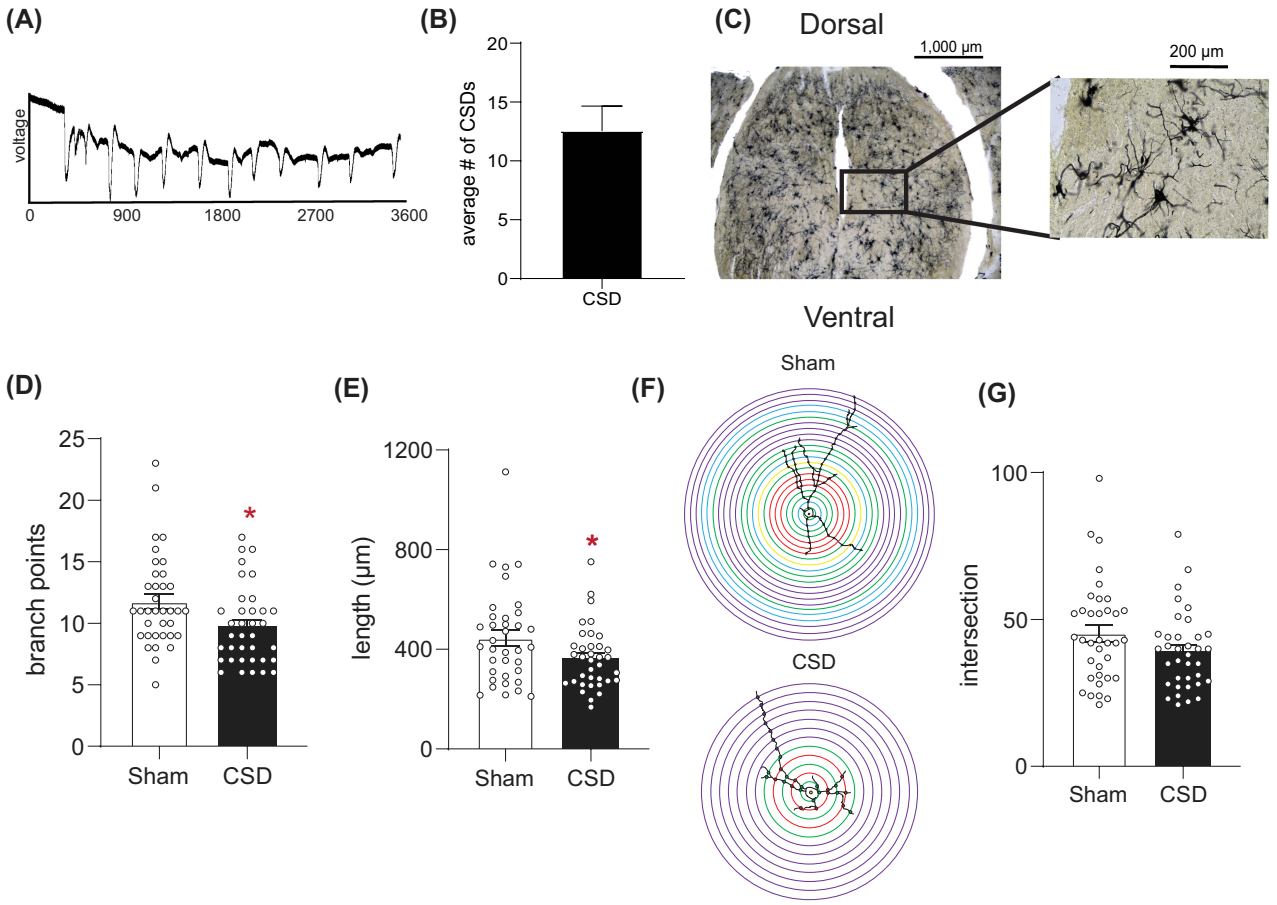


FIGURE 3 Cortical spreading depression (CSD) results in decreased neuronal complexity in the periaqueductal gray (PAG). A, Representative line tracing of CSD events over a 3600 s period. B, Graph shows the average number of CSD events that occurred in the hour of recording. $n = 7$ mice. C, Representative image taken of Golgi stained PAG at 4× (left) and 20× (right). D, The number of branch points/neuron was significantly decreased in the CSD group compared to sham surgery counterparts. Unpaired *t*-test $^{*}p < 0.05$. E, Total neuron length was also found to be significantly decreased following CSD. Unpaired *t*-test $^{*}p < 0.05$. F, Representative Sholl image of neuron from sham (left) and CSD (right) groups. G, Sholl analysis revealed a decrease in interactions following CSD, but was not statistically significant. Unpaired *t*-test. $n = 6$–7/mice/group, 6 neurons per mouse.

We determined if CRPS produced similar changes in this key pain processing region (Figure 5A). Contrary to the migraine models, in CRPS we observed increased neuronal complexity in this region. Although there was no significant increase in branch points (Figure 5B), we did observe a significant increase in total neuronal length (Figure 5C, 32% increase relative to control) and complexity within the vlPAG in CRPS mice versus controls (Figure 5D,E, 29% increase relative to control).

We also determined if CRPS altered neuronal complexity in pyramidal cells in the SCx (Figure S1A in supporting information), as we observed significant decreases in this region following chronic NTG or CSD.[15] No change was observed in branching (Figure S1B), length (Figure S1C), or complexity (Figure S1D,E). These data indicate that different brain regions show distinct cytoarchitectural alterations in response to CRPS.

## DISCUSSION

Our results build on previous studies that support the notion that chronic pain is characterized by alterations in neuronal plasticity. A summary of the cytoarchitectural changes in this study and our previous work can be found in Table 1. We continued this work by first examining alterations following the chronic NTG model.[34] NTG has long been used as a human migraine trigger and has been used experimentally to induce migraine attacks in humans and migraine-like symptoms in rodents.[47] NTG produces delayed allodynia, photophobia, and altered meningeal blood flow in mice[48–50] and results in activation of nociceptive pathways.[51,52] We previously demonstrated that chronic NTG decreased cytoarchitectural complexity in many brain regions important for migraine processing including the TNC, SCx, and the PAG.[15]

We built on these previous findings by revealing alterations in the VPM of the thalamus. Interestingly, in stark contrast to the decreased neuronal complexity observed within the TNC, PAG, and SCx, we saw an overall increase in the number of branches within VPM neurons. There are known dura-sensitive neurons within the VPM that receive direct projections from the TNC.[37] The VPM was shown to become sensitized following repeated activation of the trigeminocervical pathway.[38] Following sensitization innocuous stimuli

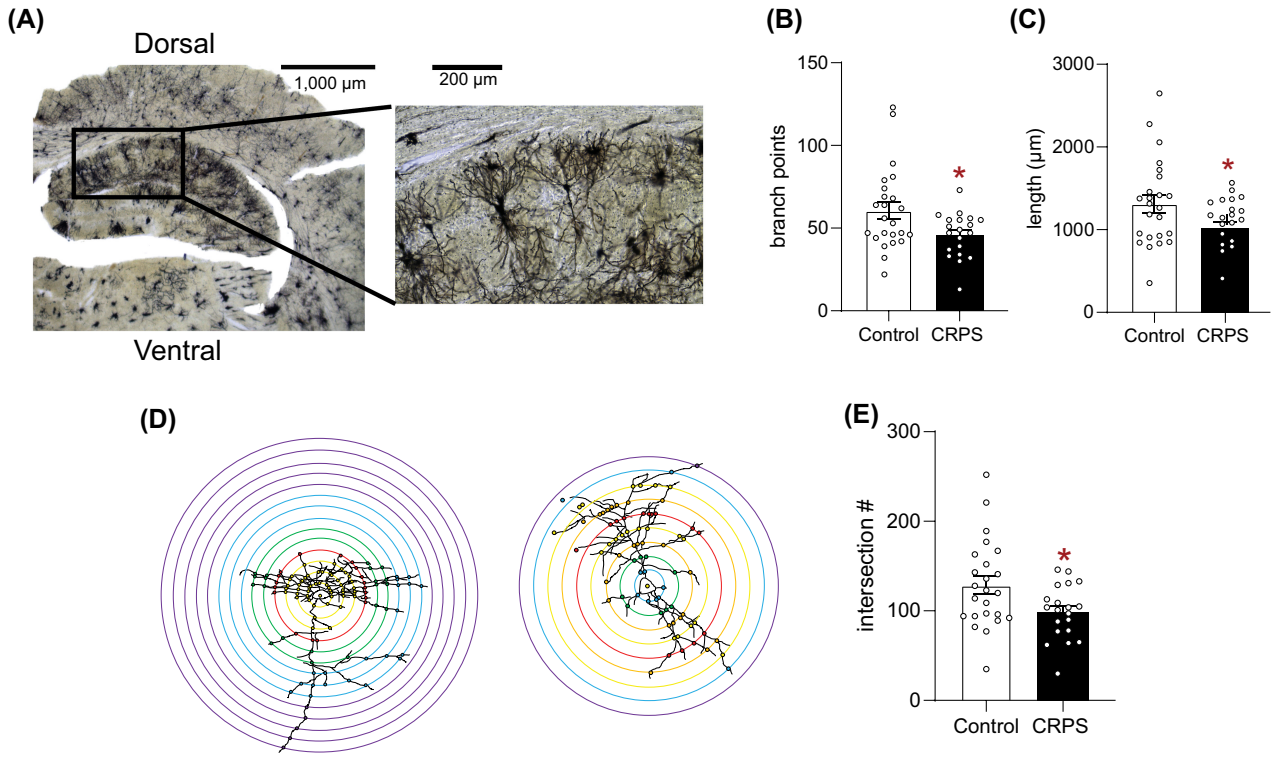


**FIGURE 4** Complex regional pain syndrome (CRPS) results in decreased neuronal complexity in the hippocampus. A, Representative image taken of the dorsal hippocampus at 4× (left) and 20× (right). B, Number of branch points per neuron were analyzed and found to be significantly decreased in the CRPS group. $^{*}p < 0.05$, unpaired *t*-test. C, Similarly, total neuron length was also significantly decreased in the hippocampus following CRPS. $^{*}p < 0.05$, unpaired *t*-test. D, Representative neurons from control (left) and CRPS (right) groups. E, Sholl analysis also revealed a significant decrease in the number of intersections following CRPS. $^{*}p < 0.05$, unpaired *t*-test.

produced activation to the level of noxious stimuli within the VPM, indicating this region is important for driving allodynia.[38] Increased branching within the VPM could regulate the allodynia associated with chronic migraine. Increased volume changes within the thalamus have been observed in humans with chronic migraine.[17,53] One study found patients with chronic migraine had increased left thalamus size and that this size positively correlated with the frequency of migraine attacks.[53] Similarly, in medication overuse headache the whole thalamus and each subnuclei also had increased volume.[17] While increased volume in humans cannot be directly compared to alterations in branching, our data are in line with the idea that augmentation of thalamic nuclei could be a driving mechanism for chronic migraine. Additionally, given our previous observations of decreased complexity in the TNC, PAG, and SCx these data suggest an overall imbalance in the migraine brain. Decreased branching from TNC could result in disruption of the usual connection between the TNC, VPM, and SCx. This could explain why we see increased complexity within the VPM, but see decreased complexity within the TNC and SCx. Future studies will be needed to better understand the connections between these regions and how changing cytoarchitecture in one region affects projections in another.

The amygdala has been implicated in regulating the affective aspects of pain and more recently to also have a role in regulating analgesia.[54] Furthermore, human imaging studies of patients with migraine have shown alterations in the amygdala, as whole brain functional connectivity of the amygdala was increased.[54] Patients with chronic migraine were also found to have decreased left amygdala volume.[55] Based on these studies we investigated if chronic NTG would also produce changes in neuronal cytoarchitecture of the amygdala. While our studies did not reveal any significant alterations in the central amygdala following chronic NTG, it is possible that functional alterations in other amygdala nuclei may occur.

The caudate putamen is a major site of cortical and subcortical input into the basal ganglia.[56] While the caudate putamen is frequently seen activated during pain, it is likely due to its role in motor function.[57,58] However, more recent findings suggest it is important in processing the sensory aspects of pain.[59] Functional imaging of the brain of patients with migraine showed decreased activation within the caudate putamen following non-repetitive stimuli.[41] Alterations in caudate signaling show the inability of the migraine brain to properly habituate itself to stimuli.[26,41] Interestingly, researchers also found increased gray matter density in the caudate of patients with migraine compared to healthy controls.[41] In our study we did not observe any alteration in neuronal complexity within the caudate. In our previous work, we also did not observe any changes in the anatomically related nucleus accumbens following chronic NTG.[15] These results in combination with our central amygdala data suggest that regions important in regulating the affective aspects

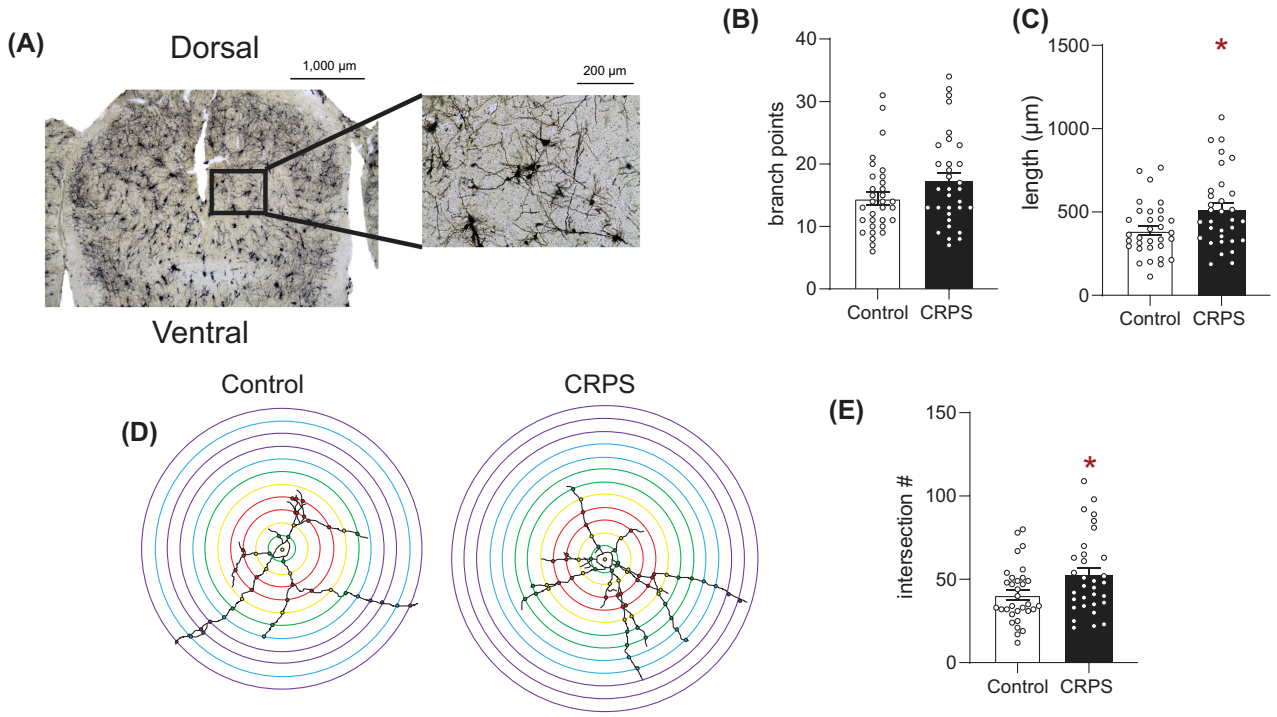


**FIGURE 5** Complex regional pain syndrome (CRPS) produces a significant increase in neuronal complexity in the periaqueductal gray (PAG). A, Representative image of the PAG at 4× (left) and 20× (right). B, Number of branch points per neuron were analyzed and no significant difference was observed between control and CRPS groups. Unpaired *t*-test. C, Total neuron length was found to be significantly increased in the PAG following CRPS. $^*p < 0.05$, unpaired *t*-test. D, Representative neurons from control (left) and CRPS (right) mice. E, Sholl analysis also revealed a significant increase in the number of intersections following CRPS. $^*p < 0.05$, unpaired *t*-test. =4/group, 8 neurons per mouse.

of pain do not undergo the same cytoarchitectural changes that we have previously observed in the TNC, PAG, and SCx.

In this study we also expanded our previous findings in the CSD model of migraine aura. CSD is thought to underlie the hyperexcitable brain state of patients with migraine, and to correlate with migraine aura.[43,60] CSD has been shown to result in neuronal swelling, alterations in dendritic structure, and even volumetric changes.[61] While CSD is primarily a cortical phenomenon it has been shown to cause sensitization of other brain regions, primarily the TNC, along with activation of meningeal nociceptors.[62,63] These data indicate that the cortical phenomenon of CSD could have other distant effects in pain circuitry. We previously found that CSD correlated with a dramatic decrease in neuronal complexity within the SCx and TNC.[15] While these two regions are the most highly implicated in CSD and migraine other connected regions may also show alterations following CSD events. The PAG is a major hub in the pain matrix and receives a direct projection from the TNC.[40,64,65] We found decreased neuronal complexity within the PAG, demonstrating that CSD events can induce neuroplastic events in pain processing regions more broadly. Activation of Pannexin1 channels has been demonstrated following CSD; and the subsequent signaling cascades are implicated in activation of trigeminal afferents promoting headache following migraine aura.[66] Furthermore, a recent study found that optogenetically stimulated CSD produced sustained periorbital mechanical allodynia as well as increased anxiety measures.[67] These studies in combination with our cytoarchitectural findings provide further support for the link between CSD and nociceptive behaviors.

We finally wanted to determine if alterations in neuronal complexity were a feature of chronic pain conditions more broadly or limited to chronic migraine. We chose to study CRPS, as this type of pain clearly transitions from acute peripheral pain to a centrally mediated chronic pain state.[22] This transition is accompanied by a host of changes including altered immune response, DNA methylation, and even some alterations in cytoarchitecture.[20,21,44,68] In patients, CRPS resulted in shrinkage of cortical mapping of the affected limb[19] and these alterations in cortical representation were positively correlated with severity of pain.[19] A previous preclinical study of CRPS showed alterations of dendritic architecture in the amygdala and perirhinal cortex.[20] This study also investigated the hippocampus and while they did not see any changes in dendrite density, they did find a decrease in synaptophysin indicating alterations in hippocampal processing.[20] A more recent study showed decreased neurite complexity within the hippocampus following injury and casting.[21] We replicated these findings, and also observed decreased neuronal complexity within the hippocampus in CRPS mice relative to controls. These results further strengthen the connection between altered hippocampal neurons and the resulting changes in cognitive function associated with CRPS.

We also found an overall increase in neuronal complexity within the vlPAG following CRPS. These findings are in sharp contrast to

TABLE 1 Summary of cytoarchitectural changes

| Model | TNC | SCx | vlPAG | Dorsal hippocampus | SC | VPM | CPu | NAc | CeA |
|---|---|---|---|---|---|---|---|---|---|
| NTG | ↓ Decrease | ↓Decrease | ↓Decrease | N/A | = No change | ↑ Increase | = No change | = No change | = No change |
| CSD | ↓ Decrease | ↓Decrease | ↓Decrease | N/A | N/A | N/A | N/A | N/A | N/A |
| CRPS | N/A | = No change | ↑ Increase | ↓ Decrease | N/A | N/A | N/A | N/A | N/A |

Abbreviation: CeA, central amygdala; CPu, caudate putamen; CRPS, complex regional pain syndrome; CSD, cortical spreading depression; SC,spinal cord; N/A, not measured; NAc, nucleus accumbens; SCx, somatosensory cortex; TNC, trigeminal nucleus caudalis; vlPAG, ventrolateral periaqueductal gray; VPM, ventral posteromedial nucleus of the thalamus.

the decreased complexity we observed in this region following both chronic NTG and repeated CSD. Considering the mechanistic differences between CRPS and migraine, these results are not surprising. One factor that could contribute to variation across models is the length of time animals underwent each condition. CSD was done over 1 h of repeated stimulation and chronic migraine exposed mice for 9 days. This is in contrast to CRPS, in which mice are in pain for 7 weeks to allow central sensitization. Additionally, CRPS begins as a peripheral chronic pain state and eventually transitions to a central one. Chronic NTG and CSD activate both peripheral and central processes from the beginning, which could cause the differences we see in cytoarchitecture. These results suggest that cytoarchitectural dynamics play a role in regulating chronic pain states, but the specific changes are unique to the type of pain.

The cytoarchitectural analysis that was conducted in these studies is limited by the Golgi staining technique, as it does not allow for co-staining with immunohistochemical markers.[69] Without this information it is difficult to determine how different cellular populations respond to chronic pain. In mice, activation of a subsection of GABAergic cells within the central amygdala was found to decrease both mechanical and thermal allodynia, while inhibition resulted in increased allodynia.[70] Our findings in the amygdala showed no significant changes. It is possible given the importance of the GABAergic subpopulation in controlling pain signals focusing solely on the GABAergic population would reveal changes in neuronal complexity that were lost when looking at gross morphological changes. Additionally, differentiation in cellular populations could give greater meaning to our cytoarchitectural changes as it could reveal more about how these changes cause alterations in signaling.

Many human anatomical studies have shown alterations in cytoarchitecture in neuropsychiatric disorders including chronic pain.[15,18,71,72] These alterations can have wide-ranging impact on the signaling of these areas and in many cases are also correlated with altered functional endpoints. Our findings give greater understanding to the possible molecular basis for these alterations. While more work needs to be conducted in the future to further investigate why some of these regions result in decreased, increased, or no change in complexity, our findings make it clear that cytoarchitectural changes are a hallmark of chronic pain. This knowledge could be used in the future to identify neuronal signatures of chronic pain states; and could result in the development of novel therapeutics targeting signaling mechanisms that govern neuronal plasticity.[16]

## AUTHOR CONTRIBUTIONS

*Study concept and design*: Zachariah Bertels, Vivianne L. Tawfik, Amynah A. Pradhan. *Acquisition of data*: Zachariah Bertels, Elizaveta Mangutov, Catherine Conway, Kendra Siegersma, Sarah Asif, Pal Shah, Nolan Huck. *Analysis and interpretation of data*: Zachariah Bertels, Elizaveta Mangutov, Vivianne L. Tawfik, Amynah A. Pradhan. *Drafting of the manuscript*: Zachariah Bertels, Amynah A. Pradhan. *Revising it for intellectual content*: Zachariah Bertels, Vivianne L. Tawfik, Amynah A. Pradhan. *Final approval of the completed manuscript*: Amynah A. Pradhan.

## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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## SUPPORTING INFORMATION

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