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Abstract

The chorda tympani is a gustatory nerve that fails to regenerate if sectioned in rats 10 days of age or younger. This early denervation causes an abnormally high preference for NH_4Cl in adult rats, but the impact of neonatal chorda tympani transection on the development of the gustatory hindbrain is unclear. Here, we tested the effect of neonatal chorda tympani transection (CTX) on gustatory responses in the parabrachial nucleus (PbN). We recorded *in vivo* extracellular spikes in single PbN units of urethane-anesthetized adult rats following CTX at P5 (chronic CTX group) or immediately prior to recording (acute CTX group). Thus, all sampled PbN neurons received indirect input from taste nerves other than the CT. Compared to acute CTX rats, chronic CTX animals had significantly higher responses to stimulation with 0.1 and 0.5 M NH_4Cl, 0.1 NaCl, and 0.01 M citric acid. Activity to 0.5 M sucrose and 0.01 M quinine stimulation was not significantly different between groups. Neurons from chronic CTX animals also had larger interstimulus correlations and significantly higher entropy, suggesting that neurons in this group were more likely to be activated by stimulation with multiple tastants. Although neural responses were higher in the PbN of chronic CTX rats compared to acute-sectioned controls, taste-evoked activity was much lower than observed in previous reports, suggesting permanent deficits in taste signaling. These findings demonstrate that the developing gustatory hindbrain exhibits high functional plasticity following early nerve injury.

New and Noteworthy:

Early and chronic loss of taste input from the chorda tympani is associated with abnormal taste behaviors. We found that compared to when the chorda tympani is sectioned acutely, chronic nerve loss leads to amplification of spared inputs in the gustatory pons, with higher response to salty and sour stimuli. Findings point to plasticity that may compensate for sensory loss, but permanent deficits in taste signaling also occur following early denervation.

Key words: Parabrachial nucleus, neural plasticity, functional plasticity, *in vivo* electrophysiology, gustatory

Introduction

The developing nervous system is highly plastic following injury. Damage to the brain at an early age is often associated with good recovery (1-5). Lost functions can be restored via the strengthening of secondary pathways prior to synaptic pruning (6-8) or the generation of new connections (9,10). Depending on the timing or type of damage, however, injury can be much more debilitating in younger animals (11,12) and plasticity following injury can sometimes be detrimental (13,14). In contrast to the central nervous system, injury to the peripheral nervous system is consistently more disruptive in developing animals (15-18).

Taste-nerve lesions can profoundly alter the development of the gustatory system. Sectioning the glossopharyngeal or chorda tympani (CT) in adult rats causes a loss of taste buds that is largely reversed following regeneration (19-22). When nerve section occurs at younger ages, taste-bud induction following regeneration is reduced (19, 23, 24). Strikingly, if the CT is sectioned (CTX) at P10 or younger in rats, the nerve fails to regenerate and there is a permanent loss of taste input from the anterior tongue (25). Bilateral CTX at P10 disrupts the typical developmental of taste-guided behaviors toward ammonium chloride (NH_4Cl) solutions (26). Neonatal rats prefer high concentrations of NH_4Cl to water, but adult rats typically do not prefer NH_4Cl at any concentration (26,27). Neonatal CTX causes adult rats to prefer hypotonic and isotonic concentrations of NH_4Cl, but preferences for NaCl develop relatively normally (26). Section of the CT in adult rats does not alter NH_4Cl preference (26,28), indicating a developmentally-dependent effect of nerve lesion.

Apart from simply removing CT input, gustatory function in the nucleus of the solitary tract (NTS) is minimally altered by P10 CTX (29), and the cause of CT lesion-induced changes in behavioral maturation is unknown. Although CTX at P10 has little influence on the development of intact gustatory nerve terminal fields, the organization of these terminal fields is altered by CTX at P5 (30). The total size of the glossopharyngeal (GL) and greater superficial petrosal (GSP) nerve terminal fields is not altered by neonatal CTX, but these nerves tend to terminate more in the dorsal NTS after P5 CT. The impact of this injury-induced change in terminal field development on central gustatory coding is unclear.

The parabrachial nucleus of the pons (PbN) receives input from the NTS and is important for stimulus identification and signaling palatability (31,32). Neurophysiological responses to NH_4Cl increase in the PbN across development (33). This developmental change is not reflected

in altered peripheral input as NH_4Cl responses are similar across development in both the CT and NTS (34,35). Perhaps not coincidentally, the shift from neonatal NH_4Cl preference to aversion occurs at a similar time scale to the change in PbN responses (27). Neonatal CTX may disrupt the development of taste-guided behaviors toward NH_4Cl by impacting the development of PbN signaling to this stimulus.

To determine the impact of neonatal CTX in the development of gustatory signaling, we recorded *in vivo* neurophysiological activity from single units within the PbN after early CTX. To ensure that gustatory responses within the PbN were devoid of CT input, we sectioned the CT in all animals prior to PbN recordings. PbN responses were compared between adult animals in which the CT was sectioned at either P5 (chronic CTX) or immediately prior to recording (acute CTX) to specifically assess neurons receiving indirect input from other gustatory nerves. We found that gustatory responses in the PbN to NH_4Cl, NaCl, and citric acid were higher in the early sectioned CTX group compared to acutely sectioned controls.

Materials and Methods

Subjects

Male (n =18) and female (n = 10) Sprague-Dawley rats were used for this experiment. Rats were bred at the University of Nebraska at Omaha vivarium with date of birth designated as P0. Upon weaning at P25, rats were housed socially in clear Plexiglas cages with corncob bedding and provided with food (Teklad) and water ad libitum. The light cycle was 12:12. All procedures were approved by the University of Nebraska at Omaha/University of Nebraska Medical Center Institutional Animal Care and Use Committee (protocol 16-096-09).

Neonatal Chorda Tympani Transection

At P5, rats were anesthetized with Brevital® (methohexital sodium; 50 mg/kg, i.p.) and the right CT was located via a small incision in the ventromedial neck. After visualization of the CT, the nerve was either cut (chronic CTX) or left intact. The incision was then closed with a single silk suture. Rats were given an injection of carprofen (5 mg/kg, s.c.) and placed on a heating pad until recovery from the anesthetic.

Parabrachial Nucleus Recordings

Surgery and Neurophysiology

After 103-283 (*M* = 174) days post-surgery, rats (240-540 g) were anesthetized with urethane (1.5-2.0 g/kg, i.p.) given in two or three doses separated by 15 min. The right tympanic membrane was punctured, and the right CT was transected with fine forceps in all rats. Even though the CT does regenerate after neonatal transection, the nerve segment proximal to injury remains in the adult tympani bulla as we have noted previously (30). This transection prior to recording ensured that no neurons in either experimental group would receive input from the CT, allowing for sampled neurons to be as similar as possible between groups (Figure 1). Animals that received CTX at P5 and immediately before recording were in the chronic CTX group, and those who received CTX only immediately before recording were in the acute CTX condition (the control group).

An incision was made in the ventral neck, and the hypoglossal and superior laryngeal nerves were transected bilaterally to prevent tongue movement and swallowing, respectively. A cannula was inserted into the trachea via an incision posterior to the larynx to facilitate breathing. A long piece of tubing (PE 205) was inserted into the esophagus via the mouth and exited an incision in the esophagus. The end of the tubing was blocked off and flanged to prevent solutions from entering the esophagus and to help open the back of the mouth, separating the posterior tongue and palate.

Rats were placed in a stereotaxic apparatus with ear bars. The position of a toothbar was adjusted until bregma and lambda were aligned in the dorsal-ventral plane. A suture placed in the tongue between the right foliate papillae and the intermolar imminence was secured so that the tongue was pulled anteriorly, medially, and ventrally to stretch open the trenches of the foliate and circumvallate papillae (36). We have also used this method previously for glossopharyngeal nerve recordings (37). A suture was inserted into the right cheek to pull it posteriorly, allowing easier access to the oral cavity.

A hole was drilled in the skull approximately 0.7 mm anterior and 1.8 mm lateral from lambda and the dura was removed. A epoxy-insulated search electrode (100-700 kΩ; FHC) was inserted into the cortex at a 20° angle with the tip pointing posteriorly to avoid the transverse sinus. A reference electrode was clamped onto nearby skin. The electrode was lowered by a

microdriver (Burleigh 6000 ULN) approximately 6-7 mm until gustatory neurons were encountered. A stimulus mixture (0.2 M NH_4Cl, 0.2 M NaCl, 0.2 M Sucrose, 0.01 M citric acid, 0.005 M quinine) was regularly applied between water rinses to identify taste-responsive cells. Neural responses to gentle stretching of the jaw via a suture around the lower incisors marked the transition from the PbN to the mesencephalic trigeminal nucleus (38). Once strong multiunit activity was identified to taste stimulation, the electrode was removed from the brain and replaced with a custom-made glass-insulated tungsten microelectrode (1-3 MΩ). Single neurons were identified by the consistency of action potential amplitude and the shape of the waveform as observed by oscilloscope and later assessed offline. In one case, responses to two neurons were recorded simultaneously. Experimenters were not blind to experimental condition during neural recordings.

Neural activity was bandpass filtered (300-1000 Hz) and amplified 1000 x (A-M Systems, 1800). Noise from 60 Hz signals were dampened with a HumBug Noise Eliminator (Quest Scientific) and amplified activity was monitored on an oscilloscope and loudspeaker. Neural activity was sampled at 20 kHz and recorded using Powerlab Chart 4.1.1 (AD Instruments). Data were analyzed offline using LabChart 8 Pro (AD Instruments).

Taste Stimuli and Stimulation

Taste stimuli were delivered through 205 PE tubing that was blocked off at the tip and punctured with holes to allow stimuli to contact taste buds in the entire oral cavity. This tubing was inserted into mouth from the anterior side so that it could be easily removed. Room temperature stimuli were delivered through the tubing by hand-operated syringe at a rate of approximately 3 ml/s over 4 s. In several rats, 0.1% methylene blue was delivered through the tubing system in this manner. In all cases, staining was observed in the circumvallate papillae, right foliate papillae, soft palate, geschmacksstreifen, nasoincisor duct, and the entire dorsal tongue surface. Although intense staining occurred in the circumvallate papillae, neurons with receptive fields in this region often exhibited delayed responses or activity that increased at the end of stimulation. This type of delay does not occur if a stimulus delivery pipette is placed directly in the circumvallate trench (39,40). Therefore, it is likely that the circumvallate papilla was not optimally stimulated by our delivery system, as has been noted in previous reports (41,42).

Taste stimuli were reagent grade and included 0.1 and 0.5 M NaCl, 0.1 and 0.5 M NH_4Cl, 0.5 M sucrose, 0.01 M citric acid, 0.01 M quinine. All stimuli were dissolved in distilled water. See Figure 2 for an example a taste-responsive neuron. Stimuli were presented in a random order with the exception that low concentration of tastants preceded high concentrations, and different concentration of the same stimulus were not given immediately after one another. Between stimulus presentations, the mouth was rinsed with distilled water for at least 40 s. Approximately 90 s elapsed between the administration of each stimulus. After presenting all stimuli, room temperature distilled water was administered in the same manner as the taste stimuli to identify somatosensory responses.

Stimulus onset was identified with a custom-made device modeled after that described by Chang and Scott (43). Taste stimuli were applied to the stimulus delivery tube through a connected metal needle that was electrically coupled with a NOR gate. When the stimulus contacted the animal, 11 nA of current flowed through the grounded rat, triggering a 5 V output from the NOR gate. This output was sampled at 1 kHz and detected via software (PowerLab Chart 4.1.1, AD Instruments), marking the beginning of stimulus contact. Because of the high flow rate, differences in onset based on oral cavity area would be minimal.

After delivery of all stimuli, the stimulus delivery tube was carefully removed from the mouth and the receptive field was identified. A small brush coated with the stimulus mixture or (occasionally) the neuron's best stimulus was brushed against taste areas of the mouth to identify neurons responding to stimulation of three areas of the oral cavity: the nasoincisor duct (NID), posterior palate (PP, including the soft palate and geschmacksstreifen), and posterior tongue (PT, including the circumvallate and foliate papillae). In one animal, a large hole was made in the right cheek to determine whether the PT and PP could be stimulated independently. With the tongue suture pulling the tongue ventrally, the posterior tongue papillae and soft palate/ geschmacksstreifen were sufficiently distant that one could stimulate each field independently without risk of stimulating both. After identifying the neuron's receptive field, the stimulus delivery tube was replaced and, whenever possible, responses were again recorded to all stimuli.

Histology

Once the last recording was completed, negative current was injected through the electrode (-6-10 µA/7 s) to make a small lesion. The rat was then overdosed with urethane and transcardially perfused with a modified Krebs solution followed by 8% paraformaldehyde.

Brains were post-fixed in 8% paraformaldehyde for at least a week and then cryoprotected with 30% sucrose. Using a cryostat, 50 µm coronal sections were obtained of the PbN. Sections were mounted on gelatin-coated slides and coverslipped with DPX. An experimenter blind to experimental condition identified the location of lesions using darkfield microscopy.

Assessment of Surgical Success

Following neonatal CTX, approximately 2/3rds of fungiform papillae are lost and ~80% of remaining papillae display an abnormal conical and highly keratinized morphology (24,25, 44). These effects persist up to two years after neonatal CT denervation (30). An assessment of the dorsal tongue surface is, thus, an easy and reliable way to determine that the CT was sectioned properly at P5. The dorsal surface of each tongue was stained with 0.1% methylene blue and inspected under a microscope. Based on papillae morphology, the success of surgery was verified for all rats.

Data Analysis

Statistical analyses were performed either with JASP (version 0.13.1.0) or R (version 4.0.2 with packages "effsize" and "Hmisc"). Graphs were made with GraphPad Prism, Excel, or eulerAPE (45). Data are presented as median ± 95% confidence interval (CI). For all tests, the alpha level was .05.

Spike Frequency

For each cell, the standard deviation (SD) of spontaneous activity was determined using the 10 s period prior to stimulation with each stimulus. Neurons were identified as taste-responsive if the net firing to at least one stimulus was ≥ 2.5 SD and greater than 1 spike / s (42,46). Only neurons that met this criterion were included in analyses.

The number of spikes before and after stimulus onset was divided by the number of seconds measured (10) to obtain spikes per second (sps). To measure taste-evoked activity for each neuron, the sps before the start of stimulus administration were subtracted from the sps after the beginning of taste stimulation. We included spikes recorded after tastant stimulation stimulation (which occurred over ~4 s) because firing occasionally increased after stimulus administration when neurons had a receptive field that included the circumvallate papilla. Because NaCl-elicited activity in the GL decreases in adult rats after CTX at P10 (37), we

wanted to ensure that neurons with posterior tongue input were sampled whenever possible. If the stimulus series was presented twice, net responses were averaged.

Many sampled neurons exhibited responses to all stimuli, including distilled water. Because a portion of neurons in the PbN exhibit somatosensory responses to mechanical stimulation of the oral cavity (47) or changes in temperature (48), these somatosensory responses could bias our results. For instance, a neuron that responds only to one taste stimulus, but responds to mechanical stimulation of the oral cavity would appear to be broadly tuned to tastes when in fact it is narrowly tuned if mechanical stimulation was eliminated. To limit the impact of somatosensory responses, we first identified whether neurons responded significantly to stimulation with distilled water. Stimulation with water occurred in the same mater as other tastants (~3 ml/s for 4 s) and neurons were considered water-responsive if the net firing (evoked spikes/s – baseline spikes/s) for the 5 s period after water stimulation onset was ≥ 2.5 SD of baseline activity and greater than 1 spike / s. We used the first 5 s of stimulation to measure water responsiveness to capture any potential somatosensory-related firing during stimulus administration. If a neuron was identified as water-responsive, the net activity (sps) from 0-10 s after water onset was subtracted from the net activity for each taste stimulus. Although there is some evidence that PbN respond to the taste of water (47), these cells represent a small portion of neurons in the PbN and taste responses in the periphery have only been identified in the superior laryngeal (49,50), a nerve we sectioned prior to recording.

To test whether data were normally distributed, Shapiro-Wilk tests were performed for each experimental group (acute CTX or chronic CTX) for each spike frequency comparison (spontaneous activity and all seven taste solutions). For 15/16 comparisons, the p values were less than .05, indicating insufficient evidence for a normal distribution for most groups. We used nonparametric Mann-Whitney U tests to compare firing rates between as a function of sex or experimental condition (acute CTX vs. chronic CTX). Because assessing the impact of sex or experimental condition on tastant-evoked firing rates required seven tests (one for each stimulus), the *p* values for these tests were adjusted using the Holm-Bonferroni method (51,52) for each independent variable. Cliff's delta (*d*), a non-parametric measure of effect size (53), was also calculated for each comparison. Values of *d* vary between -1 and 1, with 0 indicating that the distribution of data for each group are completely overlapping, and -1 or 1 indicating no

overlap between group distributions (54). Effect sizes may be considered small at .11, medium and .28, and large at .43 (55).

Bayes Factor (BF) was calculated for each comparison as well. BF values provide a way to help distinguish between two types of statically non-significant results: those that suggest the independent variable has no effect (evidence of absence), and those that suggest there is insufficient data to determine the efficacy of the independent variable (absence of evidence; 56). In other words, BF values indicate the strength of evidence in favor of the alternative hypothesis (experimental groups are different), the null hypothesis (no difference between groups), or situations where there is insufficient evidence to favor either hypothesis. BF values above 3 indicate a moderate evidence for the alternative hypothesis (experimental groups are different) and values above 10 suggest strong evidence for this outcome (57). BF values under 1/3 indicate moderate evidence for the null hypothesis and values between 1/3 and 3 suggest that the null and alternative hypotheses are similarly probable. Two-tailed Bayesian Mann-Whitney tests were run with JASP to determine BF values.

Identical statistical tests were performed using a subset of data that only included neurons from animals where the recording location was verified with a lesion.

Effects of Age on Neural Responses

Animal ages fit the criteria for normal distribution as assessed by Shapiro-Wilk tests. The age of animals in each experimental group was compared with a Welch's *t* test. To determine if the age of an animal was related to its neural responses, seven Pearson correlations were run between age of the animal for each neuron and the neuron's net response to each taste solution. The Holm-Bonferroni method was used to correct the *p* values for seven comparisons.

Receptive Fields

The receptive field for each neuron was identified as one or more of the following: NID, PP, or PT. Because some receptive field combinations (e.g. PP/PT) occurred in a small number of cells, receptive field organization was compared between neurons from chronic CTX and acute CTX rats by identifying the number of times each receptive field was observed. That is, if a cell from a rat in the acute CTX group responded to stimulation of all receptive fields, then

NID, PP, and PT were each listed under the acute CTX condition. Receptive field distribution was compared between experimental groups with a chi-squared test.

Entropy

To determine if a neuron's breadth of tuning was affected by neonatal CTX, we identified entropy (*H*) for each cell (58) using the following equation:

$$H = -K \Sigma p_i \log p_i$$

Where K is a scaling constant and p_i is the response for each stimulus as a proportion to responses for all stimuli. K was set to 1.431 for five stimuli: 0.1 M NH_4Cl, 0.1 M NaCl, 0.5 M Sucrose, 0.01 M citric acid, and 0.01 M quinine. An *H* value of 0 would indicate that a neuron responded to one and only one stimulus, whereas a value of 1 means a neuron responded equally to all stimuli. Because this equation cannot handle negative responses, absolute values were used. To be consistent with previous reports that did not assess responses to NH_4Cl, we also calculated entropy using four stimuli: 0.1 M NaCl, 0.5 M Sucrose, 0.01 M citric acid, and 0.01 M quinine. For this analysis, a K value of 1.661 was used. Shapiro-Wilk tests indicated that entropy data from neurons in the chronic CTX condition were not normally distributed (when calculated with four or five stimuli, each $p < .001$). Entropy was compared between experimental conditions with Mann-Whitney U tests. Cliff's *d* effect sizes and BF_{10} values are reported.

Inter-Stimulus Correlations

Pearson correlations were performed for each stimulus pair to determine if the similarity of responses between stimuli was affected by timing of CTX. Separate correlations were performed for chronic CTX and acute CTX animals. Given the large number of interstimulus correlations for each group (n = 21), we adjusted each *p* value using the Holm-Bonferroni method (51,52).

Results

General Characteristics

Gustatory responses were recorded from 25 neurons in acute CTX rats and 25 neurons in chronic CTX animals. See Figure 2 for an example of a gustatory neuron recording from a

chronic CTX rat. Information about the animals from which neural recordings occurred is shown in Table 1. From each rat, activity from one to four neurons (M = 1.8) was recorded. The age of acute CTX rats ($M \pm SEM$ = P179.1 ± 14.9) was not significantly different from chronic CTX rats (167.5 ± 12.8; Welch's t test, $t(25.86) = 0.588$, $p = .562$, Cohen's $d = .221$, $BF_{10} = 0.401$). Correlations between age and neural responses for each stimulus were not significant (all p values > .363), suggesting the time after neonatal surgery did not impact neural activity. Animal weights at the time of recording were not significantly different between groups (Welch's t test, $t(23.14) = 0.455$, $p = .653$, Cohen's $d = .177$, $BF_{10} = 0.391$), suggesting eating behavior was similar for all rats.

Significant, excitatory neural responses to water occurred during stimulation (0-5 s after stimulus onset) for 7/25 (28%) neurons in chronic CTX rats and 7/25 (28%) cells from acute CTX animals. Neural activity during water stimulation could be a somatosensory response: either the result of oral cavity mechanical stimulation (59) or changes in temperature (48). In these cases, somatosensory responses would be expected to occur for all stimuli since they were the same temperature and presented at the same flow rate. Another possibility is that water-elicited activity actually represents a taste response (47). When we tested whether neural responses to water occured after stimulus cessation (5-10 s after stimulus onset), only one neuron (cell #34) showed a significant response which was inhibitory and followed an excitatory response during stimulation. The majority of responses to water appear somatosensory and subtracting this response from taste-evoked activity creates a more accurate reflection of how the neurons respond to taste stimuli.

Spontaneous Activity

There was a non-significant tendency for male rats to have higher spontaneous activity than female rats ($W = 220.5$, $p = .089$, Cliff's $d = .28$, $BF_{10} = 1.004$). There was no significant difference in spontaneous activity between chronic CTX and acute CTX rats ($W = 385.5$, $p = .159$, Cliff's $d = .23$; $BF_{10} = 0.597$; Figure 3). Spontaneous activity was very low for many neurons, with six cells exhibiting no firing at all in the absence of stimulation.

The Effects of CTX on Taste-Evoked Activity

Neural activity in response to taste stimulation was not significantly different for male and female rats (for each taste stimulus, $p > .60$). BF_{10} values for each comparison ran between .313 and .858 ($M = .462$), suggesting insufficient evidence to determine an effect of sex on taste responses. Taste-elicited activity was higher in chronic CTX rats, but not for all stimuli. Compared to acute CTX animals, rats receiving CTX at P5 had significantly higher firing rates to stimulation with 0.1 M NH4Cl ($W = 458$, $p = .025$, Cliff's $d = .47$; $BF_{10} = 7.662$), 0.5 M NH4Cl ($W = 469$, p = .013, Cliff's $d = .50$, $BF_{10} = 12.788$), 0.1 M NaCl ($U = 444$, $p = .044$, Cliff's $d = .42$, $BF_{10} = 2.874$), 0.5 M NaCl ($W = 441.5$, $p = .039$, Cliff's $d = .41$, $BF_{10} = 3.053$), and 0.01 M citric acid ($W = 470$, $p = .013$, Cliff's $d = .50$, $BF_{10} = 6.309$; Figure 4). There were no significant differences in neural responses to stimulation with 0.5 M sucrose ($W = 330.5$, $p = 1.000$, Cliff's $d = .06$, $BF_{10} = .306$) or 0.01 M quinine ($W = 322.5$, $p = .854$, Cliff's $d = .03$; $BF_{10} = 0.293$) between experimental groups. The BF_{10} values for quinine and sucrose are less than 1/3, suggesting moderate evidence for a lack of effect of surgical condition of responses evoked by these stimuli. Evoked responses and animal information for each cell are shown in Figure 5.

Receptive Fields

The receptive field could be determined in 21/25 (84%) neurons from chronic CTX rats and 18/25 (72%) of cells from acute CTX rats. In some cases, the cell was lost before the receptive field could be identified and, for others, the evoked response to brush stimulation was not clearly distinguishable from baseline activity. The receptive field information for each surgical group is displayed in Figure 6. The occurrence of each receptive field (NID, PP, PT) was not significantly different between surgical groups (χ^2 (2, N = 64) = 1.559, $p = .459$). No responses to anterior tongue stimulation were observed.

***E**ntropy*

Entropy could not be calculated for one neuron from a chronic CTX rat (cell 50) because the cell had no spontaneous activity an no evoked activity for any stimulus except 0.5 M NH_4Cl. This cell was excluded from the entropy analysis. When entropy was calculated using the responses to five stimuli (including 0.1 M NH_4Cl), neurons from rats receiving CTX at P5 has significantly higher entropy compared to neurons from acute CTX rats ($W = 410$, $p = .028$, $BF_{10} = 1.344$; Figure 7A). Similar results were found when four stimuli were used to assess entropy

(Figure 7B). Neurons from chronic CTX rats had significantly higher entropy than those from acute CTX rats (W = 411.5, p = .026, BF_{10} = 1.721). These findings suggest that compared to adult rats in which the CT was lesioned acutely, rats given CTX at P5 has more broadly tuned gustatory PbN neurons. However, these findings should be interpreted cautiously given the low Bayes Factor values (BF_{10} between 1/3 and 3) indicating weak evidence for the alternative hypothesis (56,57).

Correlations Across Stimuli

Inter-stimulus correlations are displayed for acute CTX and chronic CTX animals in Table 2. Compared to acute CTX animals, rats given CTX at P5 had higher correlations between stimuli, and 18 out of 21 stimulus pairs were significantly correlated. This higher correlation between stimuli for experimental animals is consistent with the larger breadth of tuning following chronic CTX but may also reflect the low evoked responses to many stimuli in acute CTX rats.

Histology

The location of neural recordings could be identified via a lesion in the majority of animals (17/28, 61%). In two brains from the P5 CTX group, the lesion was not identifiable in the sectioned tissue. In other cases, a lesion was not made because additional neural recordings were attempted, but not successful. Six lesions were reconstructed from chronic CTX rats and 11 were identified in acute CTX animals (Figure 8). The majority of recording sites were inside or adjacent to the brachium conjunctivum. Although the brachium conjunctivum is not actually part of the PbN, it is very common to encounter gustatory neurons in this region (33,38,42,60-62), and we refer to these as PbN neurons to be consistent with the literature. Lesions from acute CTX rats, but not from chronic CTX animals, were found in the rostral-most gustatory PbN. Given the smaller sample of lesions from CTX rats, it is unclear whether this difference was consistent between groups. There is some anatomical organization by taste quality in the PbN (48,63), but not in the rostral-caudal axis. We found no lesions in the medial PbN, despite this region containing many taste-responsive neurons (31,64). A majority of neurons in the medial PbN respond best to stimulation of the anterior oral cavity, including the anterior tongue (59). Since the CT was transected prior to recording in all animals, it is possible that activity in the

medial PbN was greatly diminished, making it harder to find taste-responsive neurons in that region.

Because recordings were historically verified less often in chronic CTX rats and recording locations did not overlap completely between groups, we sought to determine whether tastant-evoked responses in rats with a verified lesion were representative of data from all rats. To accomplish this, we performed statistical analysis like those described for the entire data set using data only from animals in which the recording location was verified with a lesion. Comparisons were made using data from 21 neurons recorded from 11 acute CTX rats and 12 neurons recorded from six chronic CTX animals. Spontaneous activity was significantly higher in cells from chronic CTX animals compared to acute CTX rats (W = 182.5, p = .036, Cliff's d = .45, BF_{10} = 1.617). Compared to neurons from animals with acute CTX, cells from chronic CTX rats had significantly higher responses to 0.1 M NH_4Cl (W = 199.5, p = .033, Cliff's d = .58, BF_{10} = 4.498), 0.5 M NH_4Cl (W = 219, p = .002, Cliff's d = .74, BF_{10} = 20.761), 0.1 M NaCl (W = 195.5, p = .040, Cliff's d = .55, BF_{10} = 3.691), and 0.5 M NaCl (W = 199.5, p = .033, Cliff's d = .58, BF_{10} = 5.929). There was a non-significant tendency for responses to 0.01 M citric acid to be higher in acute CTX rats compared to those in the chronic CTX condition (W = 189.5, p = .054, Cliff's d = .50, BF_{10} = 1.816). There was no significant difference between experimental groups in neural responses to 0.5 M sucrose (W = 156.5, p = .522, Cliff's d = .24, BF_{10} = 0.506) or 0.01 M quinine (W = 140.5, p = .600, Cliff's d = .12, BF_{10} = 0.381). There was a non-significant tendency for Entropy to be higher in chronic CTX rats compared to acute CTX animals when assessed with either five stimuli (W = 175, p = .069, Cliff's d = .39, BF_{10} = 0.886) or four stimuli (W = 174, p = .075, Cliff's d = .38, BF_{10} = 1.059). These findings are very similar to those using the entire data set, suggesting that the observed differences in neural responses between groups are related to differences in PbN activity. See Figure 5 for gustatory responses for all neurons with or without a verified lesion.

Discussion

Early CTX alters the development of gustatory responses in the PbN. Compared to adult animals in which the CT was transected immediately prior to recording, adult rats given CTX at P5 had higher tastant-evoked firing rates to NH_4Cl, NaCl, and citric acid. There was no significant difference in PbN activity to sucrose or quinine stimulation, suggesting that altered

responses are stimulus specific. After CTX at P5, PbN cells had higher breadth of tuning and larger across-neuron correlations. Together, these results suggest that the developing taste system is highly plastic and may respond to early sensory loss by amplifying spared inputs.

Apart from simply removing CT input, CTX at P10 has little effect on gustatory responses in the NTS (29), suggesting that neonatal CTX has minimal impact on gustatory responses in PbN-projecting NTS neurons. One possible explanation for the present results is that early, chronic CTX alters the structure of ascending inputs from the NTS. Similar to our findings, there is higher tastant-evoked firing, inter-stimulus correlations, and breadth of tuning in the NTS compared to peripheral nerves (34,35,65-71, reviewed in 72). These changes appear to be caused by convergence of peripheral nerve fibers onto NTS neurons (29,36,73,74). A similar increase in convergent afferents could occur from PbN-projecting NTS neurons following a chronic loss of CT input. This hypothesis could be tested by injecting small numbers of palatal taste buds (75) with a transsynaptic neural tracer such as pseudorabies virus (76) after P5 CTX. If this model is correct, one would expect that the ratio of labeled PbN neurons to labeled NTS neurons would be higher in neonatally transected animals compared to controls.

Another potential cause of the differences we observed between surgical groups is changes in top-down inputs to the PbN following chronic CTX. The majority of PbN neurons receive input from the lateral hypothalamus, amygdala, or gustatory cortex (77-79). Activation of these forebrain regions typically leads to inhibition of gustatory responses in a way that reduces "side band" interference and leads to a reduced breadth of tuning (77,78). Removal of signals from the central amygdala via bilateral lesion increases PbN responses to some taste stimuli (80). It is possible that a reduction in top-down signals could occur after chronic CTX, facilitating gustatory responses in the PbN. Although stimulating the amygdala decreases entropy and interstimulus correlations in gustatory PbN neurons (77,80), lesioning the amygdala does not alter breadth of tuning or correlations between stimuli (80). Additional experiments would be needed to determine how afferent and efferent inputs to the PbN change following chronic CTX.

In the current study, we decided to use acute CTX rats as controls rather than intact animals. If we wanted to directly compare the responses of neurons without CT input using intact rats, we would need to sample a large portion of neurons without a receptive field on the anterior tongue or foliate papillae, both regions innervated by the CT (81). Roughly 15% of NTS neurons meet this strict criterion (29,82) and a similar amount would be expected in the PbN (59). Using

control rats in which the CT was cut immediately before recording allowed us to sample neurons that receive input from taste buds innervated by the GL and GSP, ensuring that neurons were as similar as possible between groups. One potential concern with our control group is that cutting the CT in acute CTX rats prior to recording could have possibly altered gustatory responses beyond simply removing CT input. Anesthetizing the CT decreases taste responses from NTS neurons not innervated by the CT (82), but this change is small in magnitude (~17%) and only noted when the whole mouth is stimulated; no effect of CT anesthesia was found when the foliate papillae, nasoincisor duct, or circumvallate papilla were stimulated individually. Thus, removing signals from the CT is unlikely to have much impact on afferent signals from the NTS. Immune responses following CTX reduce amiloride-sensitive NaCl responses in the contralateral CT one day post-surgery (83,84), raising the possibility of functional changes related to immune responses after acute CTX. However, taste buds innervated by the GL have little to no amiloride-sensitive NaCl responses (37,85,86) and immune responses in the NTS after CTX do not occur until two days after surgery (87). The few hours between acute CTX and neural recordings are unlikely to be of sufficient duration for the injury-induced immune response to impact PbN function. We believe the time between acute CTX and neural recordings was too brief for changes in neural function other than CT removal to occur.

Developing rats shift from preferring high concentrations of NH_4Cl and NaCl as neonates to avoiding NH_4Cl and hypertonic NaCl concentrations as adults (27). Neonatal CTX disrupts the development of NH_4Cl aversion but has minimal impact on how NaCl preferences develop (26). Despite the profound difference in behavioral development between these stimuli following early CTX, we found that responses to NH_4Cl and NaCl were both enhanced after P5 CTX. However, unlike rats with an intact CT (33), PbN responses to NH_4Cl were consistently higher than activity to NaCl. The PbN typically contains a large number of sodium-best neurons that respond well to hypotonic NaCl (77, 79) at concentrations that are highly palatable to rats (26,88). Because PbN neurons are important for signaling palatability (31,32), it is possible that neurons that would normally signal preferred concentrations of NaCl are repurposed for NH_4Cl signaling following early CTX. It would be informative to assess whether similar changes in PbN function occur after chronic CTX in adult rats. Although changes in NH_4Cl preference do not occur after adult CTX (26,28), we cannot know with certainty that the plasticity we observed in the current study is developmentally related.

Differences in neural responses between acute CTX and chronic CTX conditions open the possibility that the brain compensates for early CT loss. However, the increase in tastant-evoked responses following chronic CTX that we observed in the present study does not appear to restore normal gustatory signaling. Compared to PbN responses from controls rats from previous studies (33,61,89), the mean responses to NaCl, NH_4Cl, and sucrose are lower in rats given chronic CTX. Responses to NaCl after chronic CTX are particularly low, with 0.1 M NaCl eliciting an of average 2.9 spikes/s in the present study vs. ~24 spikes/s in urethane-anesthetized, intact rats (89). Furthermore, the ability of the PbN to discriminate between stimuli appears to be impaired in chronic CTX rats. Adult CTX impairs detection and discrimination of salts (90-93). This impairment is consistent with our finding that NH_4Cl and NaCl responses were significantly correlated in acute CTX rats, suggesting the PbN responds similarly to these salts. If changes to PbN function after chronic CTX were compensatory, one would expect interstimulus correlations to reduce, allowing the PbN to better distinguish between taste stimuli. However, correlations between NaCl and NH_4Cl are higher in the chronic CTX group and the responses to 18/21 stimulus pairs are significantly correlated. Although partial compensation for CT loss may occur, chronic CTX rats appear to have permanent deficits in gustatory signaling. Given our findings, a more systematic study of taste-guided behavior following early CTX would be revealing.

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Figure 1. Diagram of the experimental design showing a rat's head as viewed from the side. At P5 the CT was accessed through the neck and either cut (chronic CTX group) or left intact (acute CTX group). The location of this surgery is shown with scissors. After 100+ days, PbN recordings were performed. Prior to recording the superior laryngeal nerve was transected, and the CT was cut in the middle ear of all animals (each cut shown with a red X). A microelectrode was then inserted into the PbN to perform extracellular recordings.

Figure 2. Gustatory activity from a single PbN neuron from a male chronic CTX rat. This neuron's receptive field was in the nasoincisor duct (NID) only. The dashed blue line indicates stimulus onset. This neuron did not exhibit a significant somatosensory response to water stimulation.

Figure 3. Spontaneous activity 10 s prior to taste stimulation. Each dot represents the baseline activity from a single neuron. Bars indicate median ± 95% CI. The number of neurons is noted for each group (n). Spontaneous activity was compared between experimental groups using a Mann-Whitney U test. There was no significant difference between surgical groups.

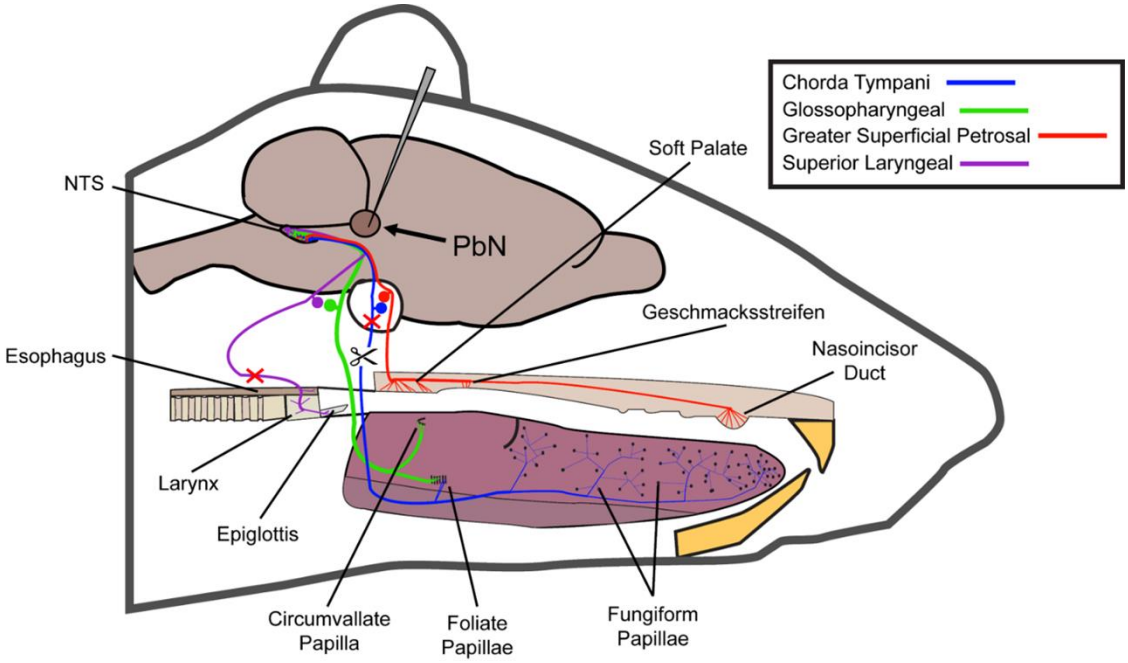
Figure 4. Net spikes over 10 s following taste stimulation for all sampled PbN neurons. All rats received CTX immediately before recording. Those in the chronic CTX group received CTX at P5 as well. Each dot represents the response of a single neuron and the number of neurons is shown (n). Bars display median ± 95% CI. Net spikes were compared between experimental groups for each stimulus using Mann-Whitney U tests with Holm-Bonferroni corrections for seven comparisons.
* $p < .05$

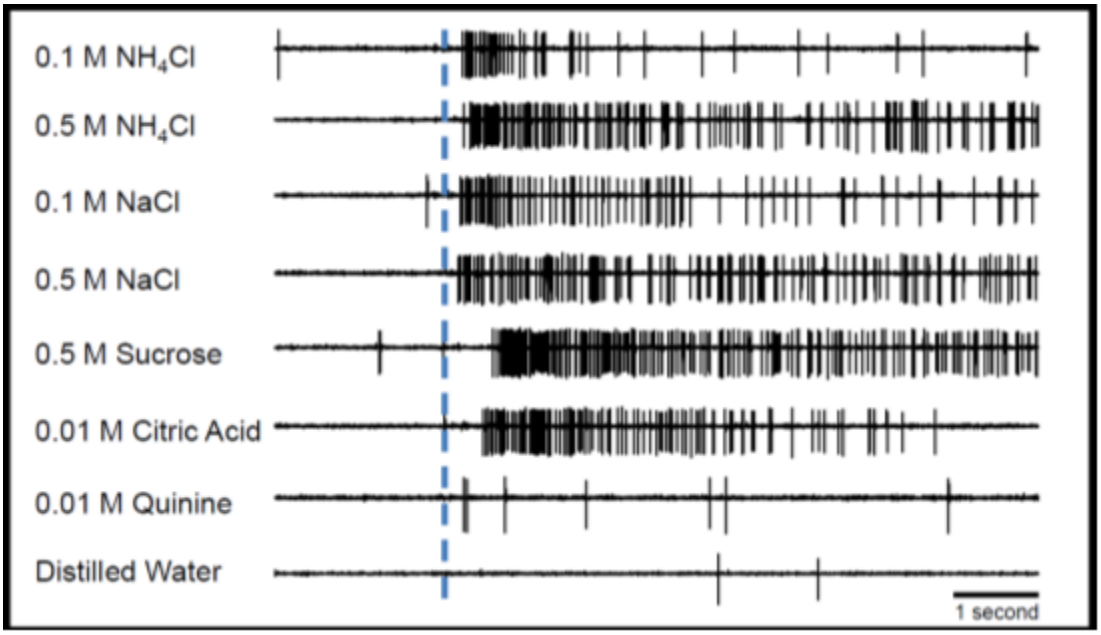
Figure 5. Net activity evoked by all stimuli for neurons from acute CTX rats (top) and Chronic CTX rats bottom. Cells are ordered by their response to 0.5 M NH_4Cl. Below the cell number, one can find the sex of the rat, whether the recording location was verified with a lesion (Y = yes, N = No), and the postnatal age (in days) of the rat at the time of recording. The cell numbers displayed here correspond to those referenced in text.

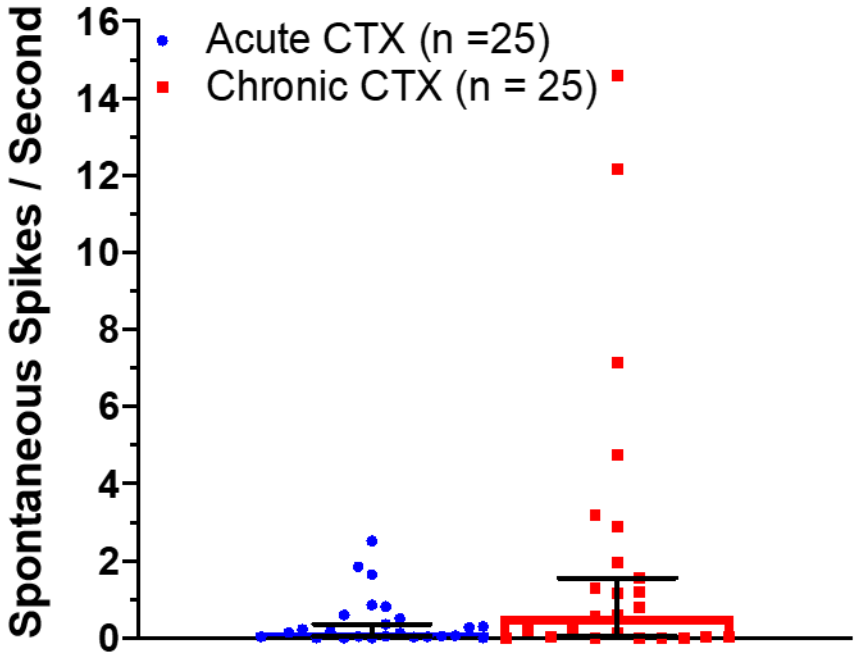
Figure 6. Venn diagram displaying receptive field information for neurons from Chronic CTX rats (n = 21) or Acute CTX rats (n = 18). The number of neurons is indicated for each receptive field. Cells for which the receptive field could not be identified are not shown. NID, nasoincisor duct; PP, posterior palate; PT, posterior tongue. This diagram was made with eulerAPE (Micallef & Rodgers, 2014). The distribution of receptive fields was compared with a chi-squared test. There was no significant difference in receptive field distribution between experimental groups.

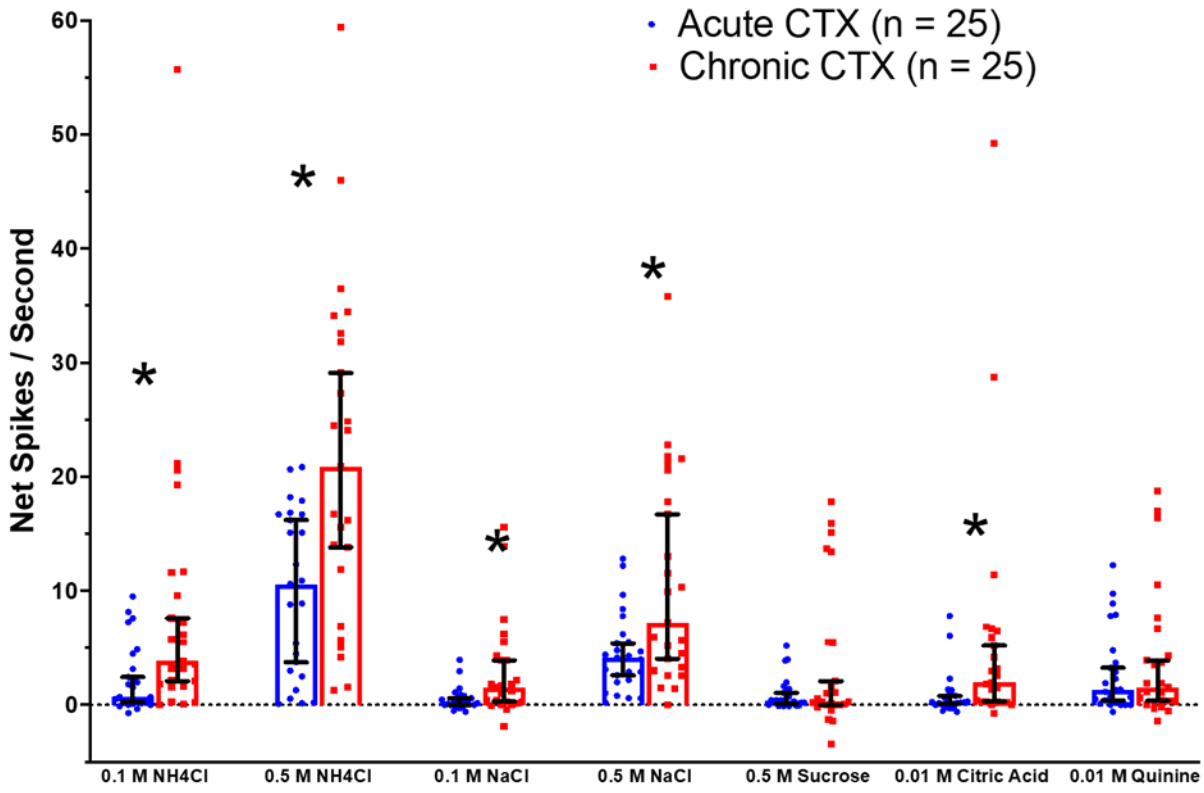
Figure 7. Entropy for neurons from acute CTX rats and chronic CTX rats. (A) Entropy values (median ± 95% CI) calculated using responses to five stimuli: 0.1 M NH_4Cl, 0.1 M NaCl, 0.5 M sucrose, 0.01 M citric acid, and 0.01 M quinine. Each dot represents the entropy value from a single neuron (B) Entropy values (median ± 95% CI) calculated using responses to four stimuli: 0.1 M NaCl, 0.5 M sucrose, 0.01 M citric acid, and 0.01 M quinine. Entropy values were compared between experimental groups using Mann-Whitney U tests.
* $p < .05$

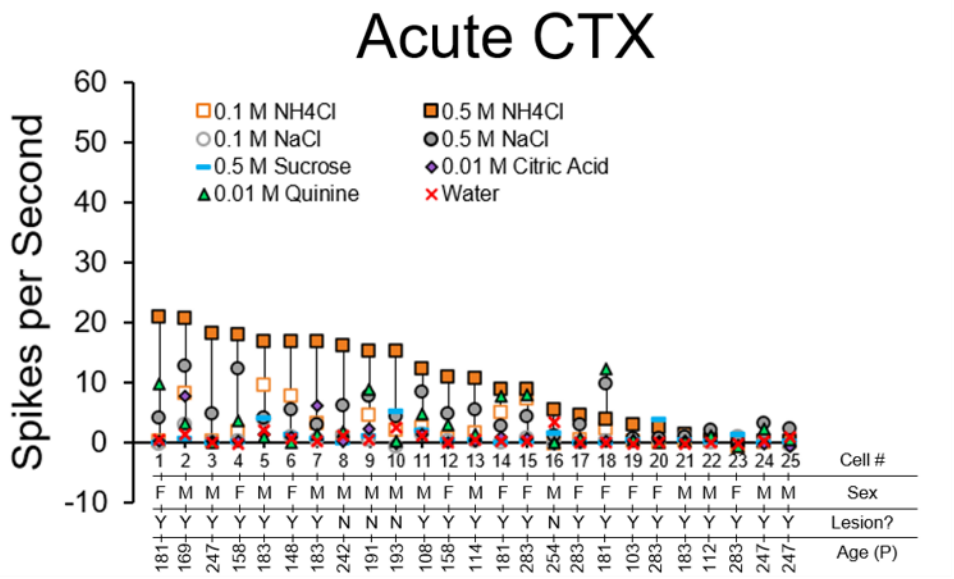
Figure 8. PbN lesions used to mark the location of neural recordings. Coronal sections after a lesion are show for the right PbN under brightfield (A) and darkfield (B) illumination. (C) Reconstruction of lesion sites in six rats from the chronic CTX group (red squares) and 11 from the acute CTX condition (blue circles) for three different locations in the PbN. The top image is the most rostral and the bottom image is most caudal. Numbers on the right refer to approximate distance from bregma in mm. BC, brachium conjunctivum; Me5, mesencephalic trigeminal nucleus; MPB, medial parabrachial nucleus; LPB, lateral parabrachial nucleus

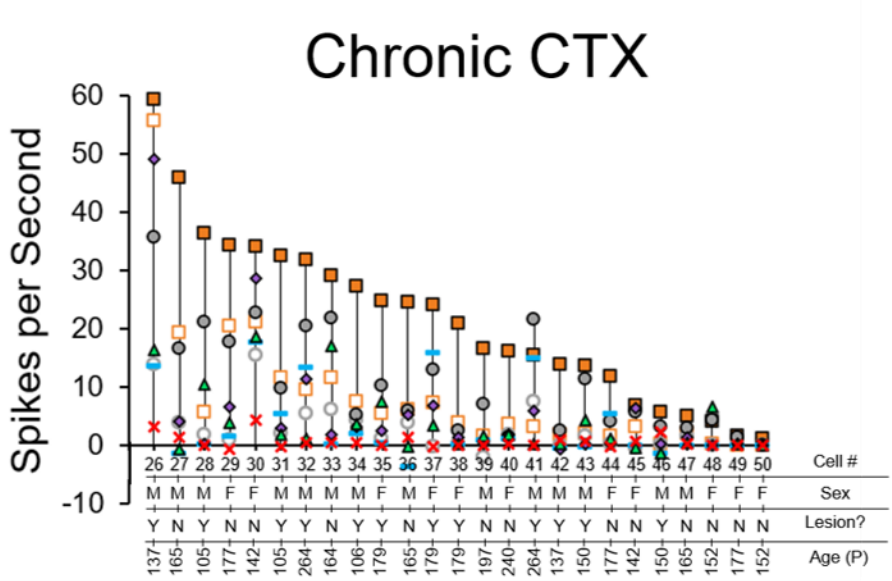

Chorda Tympani
Glossopharyngeal
Greater Superficial Petrosal
Superior Laryngeal
NTS
PbN
Soft Palate
Geschmacksstreifen
Nasoincisor Duct
Esophagus
Larynx
Epiglottis
Circumvallate Papilla
Foliate Papillae
Fungiform Papillae

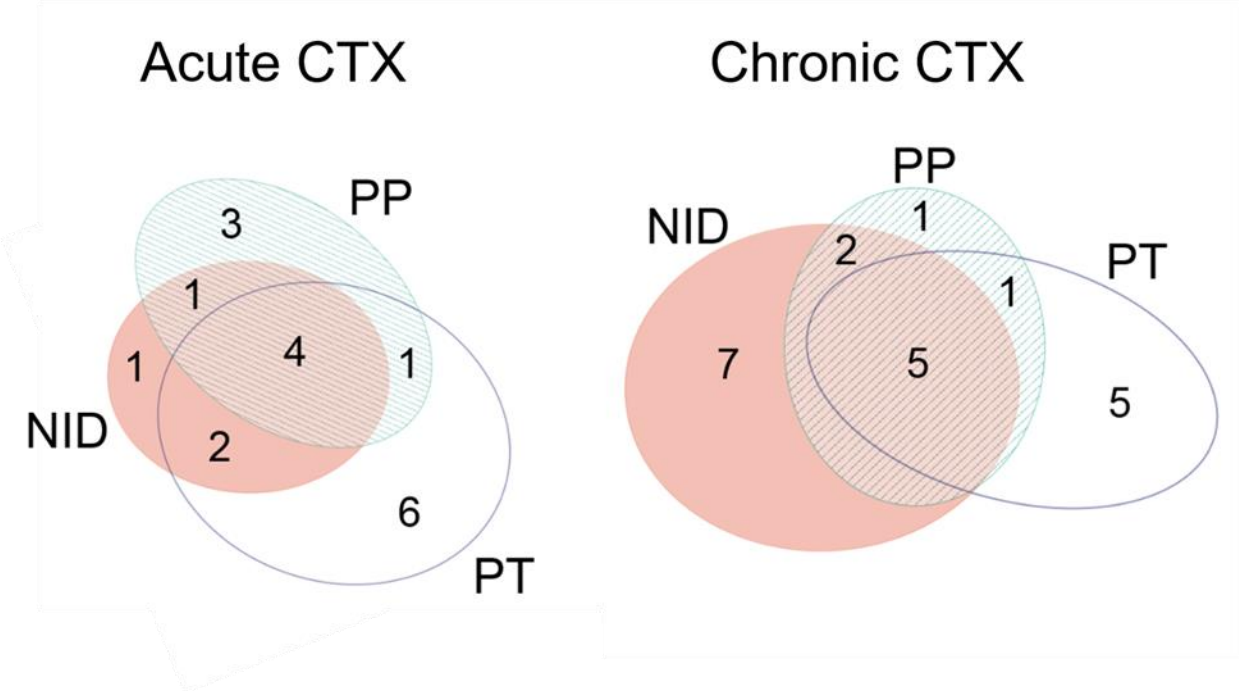

0.1 M NH_4Cl
0.5 M NH_4Cl
0.1 M NaCl
0.5 M NaCl
0.5 M Sucrose
0.01 M Citric Acid
0.01 M Quinine
Distilled Water
1 second

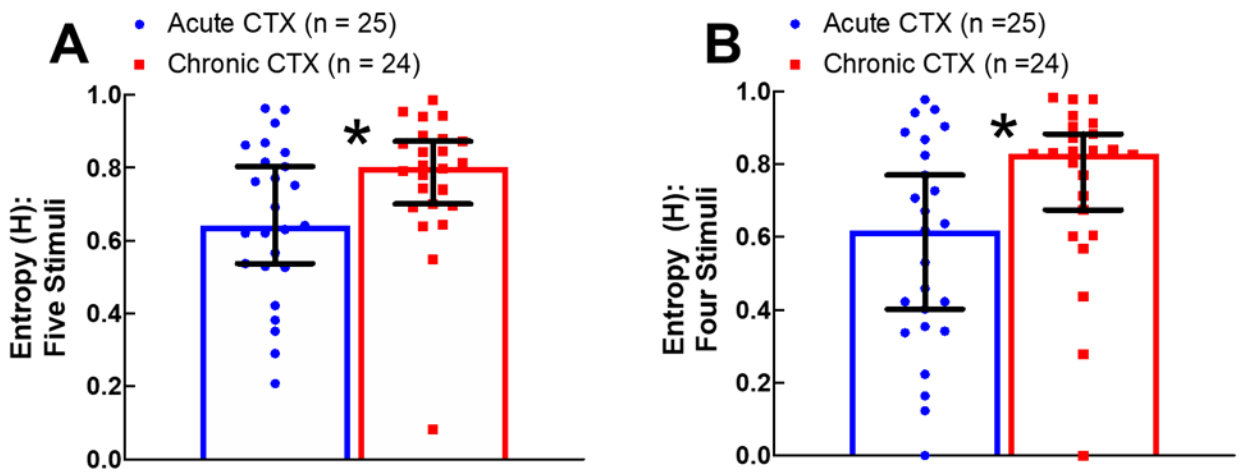

Spontaneous Spikes / Second
16
14
12
10
8
6
4
2
0
Acute CTX (n =25)
Chronic CTX (n = 25)

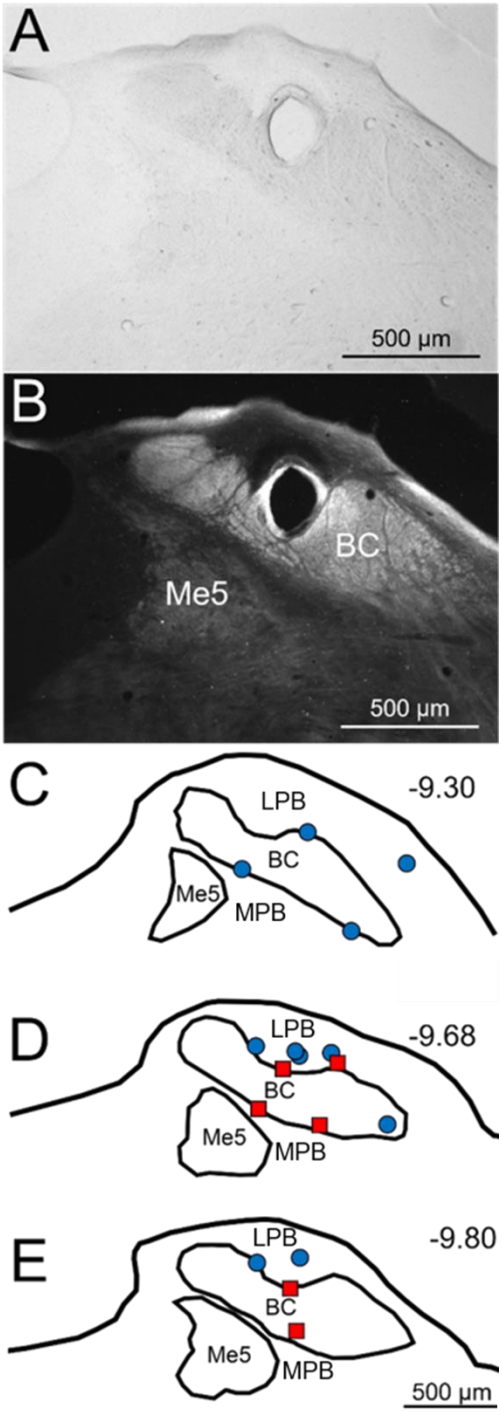

Acute CTX (n = 25)
Chronic CTX (n = 25)
Net Spikes / Second
60
50
40
30
20
10
0
0.1 M NH4Cl
0.5 M NH4Cl
0.1 M NaCl
0.5 M NaCl
0.5 M Sucrose
0.01 M Citric Acid
0.01 M Quinine


Acute CTX
Spikes per Second
60
50
40
30
20
10
0
-10
0.1 M NH4Cl
0.5 M NH4Cl
0.1 M NaCl
0.5 M NaCl
0.5 M Sucrose
0.01 M Citric Acid
0.01 M Quinine
Water
Cell #
Sex
Lesion?
Age (P)


Chronic CTX
Spikes per Second
60
50
40
30
20
10
0
-10
Cell #
Sex
Lesion?
Age (P)


Acute CTX
PP
3
1
1
4
1
NID
2
6
PT
Chronic CTX
PP
NID
1
2
PT
1
7
5
5


A
Acute CTX (n = 25)
Chronic CTX (n = 24)
Entropy (H):
Five Stimuli
1.0
0.8
0.6
0.4
0.2
0.0
*
B
Acute CTX (n =25)
Chronic CTX (n =24)
Entropy (H):
Four Stimuli
1.0
0.8
0.6
0.4
0.2
0.0
*


A
500 µm
B
BC
Me5
500 µm
C
-9.30
LPB
BC
Me5
MPB
D
-9.68
LPB
BC
Me5
MPB
E
-9.80
LPB
BC
Me5
MPB
500 µm