Functional status of the regenerated chorda tympani nerve as assessed in a salt taste discrimination task

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Kopka, Stacy L., Laura C. Geran, and Alan C. Spector. Functional status of the regenerated chorda tympani nerve as assessed in a salt taste discrimination task. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R720–R731, 2000.—We tested whether the recovered ability of rats to discriminate NaCl from KCl after chorda tympani nerve transection (CTX) is causally linked to nerve regeneration or some other compensatory process. Rats were presurgically trained in an operant NaCl vs. KCl discrimination task. Rats with regenerated nerves, histologically confirmed by anterior tongue taste pore counts and tested 62 days after CTX (CTX-62R; n = 5), performed as well as those tested 62 days after sham surgery (Sham-62; n = 5), but both of these groups initially performed slightly worse than animals tested 7 days after sham surgery (Sham-7; n = 4). Performance of rats tested either 7 (CTX-7P; n = 5) or 62 (CTX-62P; n = 4) days after CTX in which nerve regeneration was prevented was severely disrupted. Adulteration of the stimuli with amiloride, an epithelial sodium channel blocker, impaired discrimination performance in a similar dose-dependent manner in the Sham-7 (n = 2), Sham-62 (n = 5), and CTX-62R (n = 5) groups, suggesting that the functional status of the amiloride-sensitive transduction pathway returns to normal in rats with regenerated chorda tympani nerves. Performance of CTX rats without regenerated nerves (CTX-7P; n = 2; CTX-62P, n = 4) was further degraded by amiloride treatment, suggesting that taste receptors innervated by other nerves are sensitive to amiloride. In conclusion, nerve regeneration is an essential component underlying full recovery of salt discrimination function after CTX.

sodium chloride; potassium chloride; amiloride; functional recovery; animal psychophysics

The chorda tympani nerve (CT), which is a branch of the VIIth cranial nerve, innervates taste buds of the anterior tongue and is exceptionally responsive to NaCl (5, 7, 21, 50). In addition to elevating NaCl detection threshold by up to two orders of magnitude (57, 64), transection of this nerve has been shown to severely disrupt taste-guided discrimination of NaCl and KCl (62, 65, 66) and to compromise the expression of sodium appetite in rats (9, 10, 22, 39, 58). Interestingly, the transected CT displays a remarkable proclivity to regenerate, and electrophysiological research with rats, gerbils, and hamsters has shown that taste and temperature responses of the regenerated CT are similar to those of intact nerves (12, 14, 46, 47). Additionally, the single-fiber responses of the regenerated CT are also similar to those of the intact nerve in the rodent species examined (13, 14, 44, 46, 47). Such findings suggest that signals carried by the regenerated CT are essentially the same as those carried by the intact nerve. This raises the question as to whether the gustatory input arising from the regenerated CT can be interpreted usefully by central neural circuits, thus restoring taste-guided behavior. Behavioral recovery after nerve regeneration is an issue of ultimate relevance, yet, although the original electrophysiological findings are already decades old, few of their behavioral implications have been examined.

Along these lines, Barry et al. (2) showed that, after CT nerve crush, hamsters do not express a presurgically conditioned taste aversion to NaCl but can reacquire one when retrained 10–16 wk after surgery, presumably due to CT regeneration. Although no measures of anterior tongue taste bud status were taken, the expression of the reacquired aversion was abolished by the transection of the regenerated CT. Similarly, St. John et al. (66) showed that the ability of rats to discriminate between NaCl and KCl improves with the reappearance of anterior tongue taste pores after CT transection (CTX). Although these results suggest that salt discrimination performance recovers as a consequence of nerve regeneration, they logically remain correlational because St. John et al. (66) were unsuccessful in preventing CT regeneration in a control group. Therefore, the possibility that processes other than nerve regeneration per se contributed to the behavioral recovery cannot be ruled out. Although NaCl vs. KCl discrimination in rats is severely impaired after CTX (62, 66), with operant procedures other than that used in the study mentioned above, it has now been shown that such rats are partially competent in this task (65).

In other words, on average, CTX rats can perform the discrimination above chance. All of the gustatory nerves have been shown electrophysiologically to respond to NaCl and KCl (see Refs. 20, 43, 55). It is therefore possible that, after CTX, the processes underlying this remaining discriminability are strengthened to compensate for the removal of the CT input.

In the somatosensory system, central reorganizational events are known to occur after peripheral nerve injury (30, 31). For example, the topography of skin surface representation in primary somatosensory cortex changes after median nerve transection in monkeys.

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(e.g., see Refs. 40 and 41). Similar reorganizational events have been observed to occur in other sensory systems and in nonprimate species as well, and it has been hypothesized that such neural plasticity may be a general feature of the adult mammalian brain (see Ref. 31). Whether reorganizational events in the gustatory system occur after nerve transection has remained virtually unexplored. Thus the possibility that compensatory changes, unrelated to CT regeneration, are contributing to the recovery of salt discrimination performance after CTX remains a viable hypothesis. Accordingly, one important goal of the present study was to test whether the link between CT regeneration and behavioral recovery in the NaCl vs. KCl discrimination task is causal.

To test for causality, it was necessary for us to include a group of animals in which the CT was discouraged from regenerating. Any recovery of performance seen in animals in which nerve regeneration was prevented would have to be necessarily attributed to compensatory processes. Since the study by St. J ohn et al. (66), we have developed a simple surgical technique that effectively prevents CT regeneration without the use of neurotoxins or excessive interruption of the ventral neck musculature (see Methods). Use of this technique enabled us to test for behavioral recovery after a prolonged post-CTX period, during which nerve regeneration was blocked. In choosing an appropriate recovery period, we were guided by the results of St. J ohn et al. (66), who showed that rats with regenerated nerves tested starting 49 days after CTX discriminated NaCl from KCl as well as control rats with intact nerves. Exploiting the fact that, in rodents, anterior tongue taste buds undergo major degenerative changes after CTX, including disappearance of the taste pore (3, 14, 23, 26, 48, 49, 66), but then reappear upon reinnervation by the CT (14, 66), St. J ohn established a useful time course for CT regeneration in the rat, which indicated that taste pores do not appear until about 28 days after CTX. Regeneration thereafter appears to increase with time, to level off at ~1/2 the control number of taste buds at 42 days after surgery. Seventy days after CTX, taste bud number is still 1/2 that of controls (see Fig. 1 of Ref. 66), and normal salt discrimination performance is observed at 49 days after CTX. We therefore chose a recovery period of 62 days (see Methods) to ensure that taste bud reappearance reached asymptote before testing for behavioral recovery began, knowing that rats tested earlier than this time are able to demonstrate competence in salt discrimination.

Our second aim of this experiment was to investigate the status of the amiloride-sensitive sodium taste transduction pathway in rats with regenerated CTs. There are thought to be at least two taste transduction pathways for sodium salts in the rat (see Refs. 8, 16–18, 27, 45, 56, 69). One pathway, referred to as transcellular, involves entry of sodium cations through epithelial sodium channels (ENaCs) located on the apical membranes of taste receptor cells. This pathway is blockable by amiloride, is cation selective, is anion independent, and is sensitive to transepithelial voltage. The other pathway, referred to as paracellular, involves the electroneutral passage of sodium and chloride through tight junctions between taste receptor cells by which the ions come into contact with submucosal receptor sites. The paracellular pathway is much less cation selective, is anion dependent, and is unaffected by either amiloride or transepithelial voltage (see Refs. 8, 17, 18, 27, 45, 69). The response of the regenerated CT in rodents to NaCl has been shown electrophysiologically to be significantly suppressed by the ENaC blocker amiloride (29, 44).

Amiloride, which appears to be tasteless to rats (6, 28, 38), disrupts performance on an NaCl vs. KCl discrimination task in a dose-dependent manner when added to the salt solutions (63). More specifically, Spector et al. (63) found that when amiloride (100 µM) was used as a solvent, the overall performance of rats in an NaCl vs. KCl discrimination task was reduced to chance levels, due primarily to errors made on NaCl trials. As the concentration of amiloride was lowered, performance improved accordingly. Moreover, when a logistic function was fit to the dose-response curve reflecting overall performance, the point of inflection corresponded well with the inhibition constant electrophysiologically measured for the amiloride dose-related suppression of NaCl responsiveness by the rat CT. Thus we took advantage of the amiloride dose dependency of performance in the salt discrimination paradigm to use it as a behaviorally based functional assay of the status of the amiloride-sensitive sodium taste transduction pathway in rats with regenerated CTs. In other words, we wanted not only to determine whether the regenerated CT supports behavioral recovery in this task, but also to examine whether such recovered function would be disrupted in a way quantitatively similar to that in rats with intact nerves.

Our third goal was to test the hypothesis that there are behaviorally relevant amiloride-sensitive taste receptor cells that are innervated by gustatory nerves other than the CT. Although the NaCl responses in the rat glossopharyngeal nerve, which innervates the posterior tongue, do not normally appear to be affected by amiloride (19, 25, 34), recent electrophysiological data have shown some amiloride sensitivity in the greater superficial petrosal nerve (GSP) (59), which innervates the palate. The presence of amiloride-sensitive receptors in the receptor field of the GSP does not, however, necessarily mean that they contribute to all taste-guided behaviors. Although few behavioral data exist that complement these electrophysiological findings, Spector et al. (63) hypothesized the existence of amiloride-sensitive receptors in other taste receptor fields based on a comparison of the relative effects of CTX compared with amiloride adulteration on NaCl vs. KCl discrimination performance. Concurrent with the results of the present study, Roitman and Bernstein (54) have recently shown, using a sham drinking paradigm, that the CTX-induced attenuation of NaCl intake in sodium-depleted rats is further reduced by adulteration of the salt stimulus with amiloride. In other words, in CTX rats, amiloride treatment decreases the expres-
sion of a sodium appetite to a greater extent than does CTX alone. Thus it appears that amiloride-sensitive receptors innervated by gustatory nerves other than the CT can contribute to behavioral responses to sodium salts. The present experiment was designed in an attempt to explicitly extend this conclusion to operant performance on a salt discrimination task.

With the previous observations in mind, we transected the CT and allowed it to regenerate in some rats while preventing regeneration in others. The appropriate sham surgeries were also performed. The two-lever operant task previously used in our laboratory, in which water-deprived rats are trained to press one lever in response to NaCl and the opposite lever in response to KCl (65), was used to assess salt discrimination performance. The performance-disruptive effects of salt stimulus adulteration with amiloride were examined in all rats.

METHODS

Subjects

Twenty-five naive male Sprague-Dawley rats (Charles River Breeder, Wilmington, MA) weighing between 200 and 250 g at the start of training served as subjects. They were housed individually in hanging wire mesh cages and received free access to food (Purina 5001) except during training and testing sessions. Colony room temperature, humidity, and light cycle (12:12 h) were controlled automatically. Training and testing sessions occurred Monday through Friday (except where otherwise noted) during the light phase. Water bottles were removed from the home cages ~24 h before the start of the weekly session and were replaced immediately after the last session of the week. During the week, subjects received their water during daily sessions in the gustometers. Subjects were weighed daily Sunday through Friday to ensure that no animal fell below 85% of its ad libitum weight. Rats that fell below this criterion weight received 5–10 ml of supplemental water.

Apparatus

All training and testing occurred in four modified computer-controlled gustometers (61, 65). Six fluid stimuli were delivered by way of a sample spout that the rat could lick through a stimulus access slot in the side wall of the chamber. The stimuli were held in pressurized reservoirs equipped with solenoid valves calibrated to deposit ~5 µl of fluid with each lick. After each taste trial, the sample spout was automatically rotated over a funnel and rinsed with distilled water. Two levers were located on either side of the stimulus access slot, and white cue lights were positioned 4.2 cm above each lever. An additional vertically oriented drinking tube could be rotated in and out of position to provide a water reinforcer when appropriate. The sample spout was rinsed and air-blown dry during the intertrial interval (ITI). The test cages were placed in sound attenuation chambers, and white noise was present throughout each session to minimize the presence of external auditory cues produced during stimulus delivery.

Table 1. Training schedule

<table>
<thead>
<tr>
<th>Days</th>
<th>Phase</th>
<th>Time Out, s</th>
<th>Limited Hold, s</th>
<th>Stimuli</th>
<th>Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–12</td>
<td>Shaping</td>
<td>None</td>
<td>180</td>
<td>0.1 M NaCl or 0.1 M KCl</td>
<td>Constant</td>
</tr>
<tr>
<td>13–18</td>
<td>Alternation</td>
<td>10</td>
<td>15</td>
<td>0.1 M NaCl and 0.1 M KCl</td>
<td>Alternation</td>
</tr>
<tr>
<td>19–20</td>
<td>Discrimination training I</td>
<td>10</td>
<td>10</td>
<td>0.1 M NaCl and 0.1 M KCl</td>
<td>Semirandom</td>
</tr>
<tr>
<td>21–23</td>
<td>Discrimination training II</td>
<td>20</td>
<td>10</td>
<td>0.1 M NaCl and 0.1 M KCl</td>
<td>Semirandom</td>
</tr>
<tr>
<td>24–25</td>
<td>Discrimination training III</td>
<td>20</td>
<td>5</td>
<td>0.05, 0.1, 0.2 M NaCl and KCl</td>
<td>Semirandom</td>
</tr>
<tr>
<td>26–39</td>
<td>Discrimination training IV</td>
<td>30</td>
<td>5</td>
<td>0.05, 0.1, 0.2 M NaCl and KCl</td>
<td>Semirandom</td>
</tr>
</tbody>
</table>

Limited hold is the maximum amount of time allotted for a response. Rats were shaped for the opposite stimulus and lever during the final 5 shaping sessions. During alternation, a stimulus was presented repeatedly until a certain number of correct responses were made. This predetermined alternation criterion was 8 in the first session, 6 in the second, 4 in the third, 2 in the fourth, and 1 in the final 2 sessions. Stimuli were presented in randomized blocks during a semirandom schedule.

Trial Structure

The trial structure was the same as that used by St. John et al. (see Fig. 1 of Ref. 65). During each testing session, the rat was allowed to complete as many trials as possible in 40 min. Each trial began when the rat licked the sample spout, marking the beginning of the sample phase. The rat received 3 s to sample the stimulus, or 10 licks, whichever came first. The house lights in the gustometer were then extinguished, and the cue lights above the levers were illuminated, indicating the beginning of the decision phase. During this phase, the rat had 5 s to press one of the levers (limited hold). If the rat pressed the correct lever, the cue lights were turned off, the house lights were activated, and the reinforcement spout was rotated in front of the stimulus access slot, signaling the beginning of the reinforcement phase. The rat then received 10 s or 40 licks access to water reinforcement, whichever came first. If the incorrect lever was pressed, or if no response was made during the limited hold, the cue lights were turned off, and the rat received a 30-s timeout during which access to the reinforcer was denied. The reinforcement or time-out phase was followed by a 10-s ITI, after which time the house lights were reactivated and the animal could initiate the next trial.

Training Procedure

Rats were trained to press one lever after presentation of NaCl and the other lever after KCl presentation. All solutions were prepared with reagent-grade chemicals (Fischer Scientific, Orlando, FL) and distilled water. The training schedule is illustrated in Table 1.

Shaping. Behavior was shaped with either 0.1 M NaCl or 0.1 M KCl and were roughly counterbalanced for both lever and stimulus. By reinforcing successive approximations to the target response with water access (30 s or 40 licks, whichever came first), we trained rats to press the appropriate lever in response to the stimulus presented. During the final five shaping sessions, the rats were trained to press the opposite lever in response to the other stimulus (0.1 M NaCl or 0.1 M KCl).
Alternation. Both salts were included in all sessions throughout the remainder of the experiment. During alternation, the rat was required to make a predetermined number of correct responses to one stimulus and was then required to make an equivalent number of correct responses to the other stimulus (see Table 1). This alternation continued over six sessions. The stimuli were alternated after eight correct responses in the first session, six in the second session, four in the third, two in the fourth, and one in the final two sessions. These correct responses did not have to be consecutive. Also during alternation, the reinforcement time was decreased to 10 s (or 40 licks).

Discrimination training. Stimuli were delivered in randomized blocks of two or six. The limited hold was decreased from 15 to 5 s, and the time out was increased from 10 to 30 s (see Table 1).

Pre- and Postsurgical Testing

The five presurgical testing sessions were 40 min in length, with parameters identical to those of Discrimination training IV (see Table 1). We chose to use three concentrations of each salt (0.05, 0.1, and 0.2 M) to render stimulus intensity an irrelevant cue, since, at any given concentration, the two salts do not likely have the same perceived intensity. All three concentrations are above threshold in intact animals, yet presumably low enough to prevent significant excitation of trigeminal nerve fibers (32, 37, 51, 60, 67). Rats began postsurgical testing (5 sessions; identical to presurgical testing) either 7 or 62 days1 after surgery (see below).

Amiloride Testing and Water Control

After postsurgical testing, animals underwent 17 sessions of amiloride testing, which consisted of nonamiloride control days interspersed among days in which either 100, 30, 10, 3, 0.94, or 0.38 µM amiloride was the solvent for all solutions. The data for the 0.38 µM day had to be excluded from analysis because of a gustometer problem on that day of testing. Therefore, the actual amiloride concentrations used in data analysis were 100, 30, 10, 3, and 0.94 µM. The amiloride concentrations were tested in the above order, with a second 100 µM amiloride testing day concluding the testing series. Amiloride testing days were separated by either one or two control days. Testing on control days was identical to pre- and postsurgical testing. On the days amiloride was used, all six salt stimuli were made using the appropriate concentration of amiloride hydrochloride (Sigma, St. Louis, MO) as the solvent instead of distilled water. This concentration of amiloride also replaced water as the reinforcement solution. Because discrimination was likely to be impaired by amiloride, the control days were added between amiloride testing days to maintain and measure stimulus control in the task. Also, the rats were tested with 100 µM amiloride two times (one time at the beginning of the series and one time at the end of the series) to verify that there were no order effects.

After amiloride testing, a water control session was conducted to confirm that the rats had not been using any extraneous cues to guide their performance. During this session, all reservoirs were filled with distilled water and arbitrarily assigned to either the “NaCl” lever or the “KCl” lever. The session parameters were identical to those of pre- and postsurgical testing.

1 The postsurgical recovery period for the Sham-62, CTX-62R, and CTX-62P groups was mistakenly reported to be 69 days in an abstract for the Society of Neuroscience’s 28th Annual Meeting (35). The correct duration was 62 days.

Surgery

All rats were assigned to one of five surgical groups in which we attempted to equate for gustometer, body weight, and overall percentage correct responses during presurgical testing. Each group also included some rats for which the left lever was associated with NaCl and some for which the right lever was associated with NaCl. The rats were anesthetized (intramuscularly) with a mixture of ketamine hydrochloride (125 mg/kg body wt Ketaset) and xylazine (5 mg/kg body wt Rompun), with supplemental doses administered as necessary.

Fifteen rats received bilateral CTX in such a way that regeneration of the nerve was promoted in 5 animals and discouraged in 10 animals. All of these surgeries began with retraction of the soft tissue of the external auditory meatus to reveal the tympanic membrane. In the five animals in which regeneration was allowed to occur, the tympanic membrane was then punctured, and the CT was sectioned with microscissors as it disappeared behind the malleus. This group of rats began postsurgical testing 62 days after surgery (CTX-62R). In the 10 rats in which regeneration was to be prevented, the CT and entire tympanic membrane, including nearby portions of the ear canal, were cauterized, and the ossicles were left intact. This surgery results in a buildup of cerumen within the internal cavity of the bulla, which presumably acts as a physical barrier to the regenerating nerve. Five of these animals began postsurgical testing 7 days after surgery (CTX-7P), and the other five started postsurgical testing 62 days after surgery (CTX-62P). Nine control rats received sham surgery, which consisted of retraction of the auditory meatus and bilateral puncture of the tympanic membrane. Four of these control rats began postsurgical testing 7 days after surgery (Sham-7), and the other five started postsurgical testing 62 days after surgery (Sham-62).

During the course of amiloride testing, two severely impaired rats from the CTX-7P group (rats 12 and 22) formed lever biases, and thus an amiloride dose-response function could not be assessed in these animals. Also, because a solenoid valve on one of the gustometers had been mistakenly set to an incorrect value for several amiloride testing days, the data obtained from the three rats in this gustometer (rats 11, 17, and 23) were considered confounded and were removed before analysis. The resulting group sizes during amiloride testing were n = 2 for Sham-7 and CTX-7P and n = 5 for CTX-62P, Sham-62, and CTX-62R.

Histology

After all testing was complete, the rats were deeply anesthetized with pentobarbital sodium (intraperitoneally) and perfused with saline followed by 10% buffered Formalin. The tongues were removed and stored in buffered Formalin until histology was performed. At the time of histological processing, the tongues were soaked in distilled water for ~30 min, immersed briefly in 0.5% methylene blue, and rinsed with distilled water to remove excess stain. The anterior lingual epithelium (from the interdental eminence to the tip) was stripped of most of its underlying connective tissue and muscle, pressed between two glass slides, and examined under a light microscope. When tongues of rats with intact nerves are treated this way, fungiform papillae are seen as relatively large, lightly staining circular patches among a background of smaller, darkly staining filiform papillae (42, 62, 66). The taste pores are clearly indicated by a darkly staining spot near the center of each fungiform papillae. When the CT is transected in rodents, fungiform taste buds
undergo degenerative changes, including disappearance of the pore, although a small proportion remain intact, depending on species (3, 23, 26, 48, 49, 68). As a result, the majority of fungiform papillae of CTX rats lack a darkly staining spot in their center. Upon reinnervation by the CT, the taste pores reappear (14, 66) and can be identified as in the normally innervated tongue. Thus, in the present study, a person unaware of group assignment counted the number of fungiform papillae with and without pores to determine whether the tongue was innervated by the CT. Some fungiform papillae degenerate after CTX and develop ectopic filiform spines. In our procedure, which involved light microscopic analysis of the tongue surface, it is difficult to differentiate such degenerated papillae from the filiform background. Accordingly, only clearly identifiable fungiform papillae were counted. The use of the methylene blue staining procedure to assess fungiform papillae was attempted for each rat by a person unaware of group assignment. One-way ANOVAs followed by Tukey’s honestly significant difference tests (HSD) were performed on each of these measures to determine the main effects of surgery (group) and differences in group means.

Performance was evaluated using the overall proportion of correct responses as the primary dependent measure. Only trials in which a response was made (i.e., the rat pressed a lever) were used in the analyses. Performance close to 50% correct corresponded to chance performance, indicating a failure to discriminate between the two salts. Paired-sample t-tests on presurgical vs. postsurgical performance were conducted for each group. Additionally, one-tailed t-tests were used to compare mean postsurgical performance for each group vs. 0.50 (the null hypothesized value). The normal approximation to the binomial distribution was used to test whether the postsurgical performance of each individual rat differed from chance (0.50; see Ref. 11). Three-way ANOVAs (salt × concentration × time) were also conducted for each group. To test for changes in performance across sessions of a given testing phase (i.e., pre- or postsurgical testing), we conducted ANOVAs across individual sessions within each phase for each group.

Difference scores were calculated for each rat by subtracting the overall proportion of correct responses during postsurgical testing from that observed during presurgical testing. This was done as a means of quantifying surgically induced changes in performance while taking into account individual differences in presurgical discriminability. Group mean difference scores were then calculated, and ANOVAs were conducted to test the main effect of group, followed by Tukey’s HSD test of pairwise comparisons. Additionally, we broke down scores by salt and concentration so that we could examine performance to each salt separately, as well as performance at different concentrations within each salt. Therefore, group mean difference scores were also examined using two-way (group × salt) and three-way (group × salt × concentration) ANOVAs.

A curve was fit to data obtained during amiloride testing on days that amiloride was used (as opposed to control days) using the same four-parameter logistic function used by Spector et al. (63)

\[
f(x) = \frac{a - d}{1 + (x/c)^b} + d
\]

where a is a constant representing the maximum asymptote of performance determined by the mean performance during control sessions, b is the slope, c is amiloride concentration at the midpoint between maximum and minimum performance (i.e., ED50), d is the minimum asymptote of performance, and x is amiloride concentration. Curves were fit to individual animals, and group means were calculated for the Sham-7, Sham-62, and CTX-62R groups. Independent t-tests were then conducted on each parameter of the function to determine whether there were differences between the group means of the Sham-62 and CTX-62R groups. The small sample size of the Sham-7 group during amiloride testing (n = 2) precluded statistical analysis with this group.

Because rats in the CTX-7P and CTX-62P groups were already impaired on the salt discrimination task, curves could not be fit to data obtained when they were tested with amiloride. Additionally, because the CTX-7P group size was n = 2 during amiloride testing, statistical outcomes would not be meaningful for this group, and the following statistics were conducted only for the CTX-62P group. Thus, in this group, the mean performance on control days was used to indicate performance at an amiloride dose of zero. A repeated-measures one-way ANOVA was then conducted on performance across amiloride concentrations. Next, paired t-tests were used to compare performance at each amiloride concentration with that at the zero concentration.

Finally, the overall proportion correct during the water control test was analyzed using the normal approximation to the binomial distribution for each rat (11). This was conducted to determine whether the score of any animal differed significantly from chance. The statistical rejection criterion for all analyses was set at the P ≤ 0.05 level.

RESULTS

Histology

One rat (rat 6) in the CTX-62P group had a high number of pores (97 pores) and a high percentage of fungiform papillae with pores (70.8%), suggesting that the anterior tongue was reinnervated by the CT. Consequently, all data obtained from this rat were removed before data analysis, resulting in n = 4 for the CTX-62P group. Also, one rat (rat 19) in the CTX-7P group had a moderate number of pores (46 pores) and percentage of fungiform papillae with pores (27.8%). However, research indicates that some taste pores and papillae do remain after CTX (1, 26, 53, 66, 68). Based on such findings and the fact that the behavior of this animal fell well within the range of behavior for rats in the CTX-7P and CTX-62P groups, we decided to include data collected from this animal in our analyses. We did, nonetheless, perform the analyses after removing these data as well, and any important differences are noted.

The mean number of pores, fungiform papillae, and percentage of papillae with pores for each group are shown in Table 2. One-way ANOVAs indicated significant effects of surgery on all of these measures (all P values < 0.001). Tukey’s HSD comparisons revealed no differences between the Sham-7, Sham-62, or CTX-62R...
groups on any of the three measures. The CTX-7P group differed from the Sham-7, Sham-62, and CTX-62R groups both on number of pores and percentage of papillae with pores (all \( P \) values < 0.001) but did not differ significantly from any of these groups on total number of papillae. Significant differences were found between the CTX-62P group and the Sham-7, Sham-62, and CTX-62R groups on all histological measures (all \( P \) values < 0.012), except for number of fungiform papillae for which there was no significant difference between the CTX-62P and CTX-62R groups (\( P = 0.161 \)). There was a significant difference between the CTX-7P and CTX-62P groups only for total number of papillae (\( P = 0.025 \)). When the data for rat 19 (see above) were removed and the analyses were repeated, this difference was no longer significant, but the difference between these two groups on the percentage of fungiform papillae with pores became significant at \( P = 0.001 \).

These results strongly suggest that the CT was fully transected in the CTX-7P and CTX-62P animals and that attempts to prevent its regeneration in these animals (rat 6 notwithstanding) were successful. Because the peripheral processes of remaining intact taste axons from other nerves apparently do not sprout (1, 33), the reappearance of taste pores in the CTX-62R animals indicated that the CT regenerated.

Pre- and Postsurgical Testing

Means for overall proportion correct during pre- and postsurgical testing for all groups are shown in Fig. 1. Significant differences between pre- and postsurgical testing were found for all groups (all \( P \) values < 0.018) except Sham-7. Additionally, one-tailed t-tests for each group comparing postsurgical mean performance with 0.50 (the null hypothesized value) indicated significant differences from 0.50 for the Sham-7, Sham-62, and CTX-62R groups (all \( P \) values < 0.001). The mean postsurgical performance of the CTX-62P group was statistically different from 0.50 (\( P = 0.026 \)), whereas mean performance of the CTX-7P group was not significantly different from 0.50 (\( P = 0.07 \)). The normal approximation to the binomial distribution showed that the postsurgical performance of every rat in the Sham-7, Sham-62, and CTX-62R groups was significantly different from chance (all \( P \) values < 0.05). This test also showed that postsurgical performance of two of the five rats in the CTX-7P group and three of the four rats in the CTX-62P group differed significantly from chance (\( P < 0.05 \)).

Figure 2 shows that individual animal performance was not strongly correlated with taste pore number. Neither the correlation between taste bud number and performance for the rats without CT nerves (\( P = 0.28 \)) nor that for rats with CT nerves (\( P = 0.63 \)) was significant. In summary, these results indicate that postsurgically, rats in the Sham-7, Sham-62, and CTX-62R groups performed well on the discrimination task, whereas rats in the CTX-7P and CTX-62P groups were impaired to various degrees. The results also indicate

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Table 2. Group numbers of pores, fungiform papillae, and percent of papillae containing pores

<table>
<thead>
<tr>
<th>Group</th>
<th>Pores</th>
<th>Fungiform Papillae</th>
<th>Percent of Papillae with Pores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-7</td>
<td>144.5 ± 10.47</td>
<td>153.0 ± 11.11</td>
<td>94.4 ± 0.44</td>
</tr>
<tr>
<td>CTX-7P</td>
<td>124.2 ± 9.56</td>
<td>144.2 ± 9.56</td>
<td>7.8 ± 5.03</td>
</tr>
<tr>
<td>CTX-62P</td>
<td>14.5 ± 2.25</td>
<td>96.8 ± 7.74</td>
<td>15.25 ± 2.58</td>
</tr>
<tr>
<td>CTX-62R</td>
<td>120.0 ± 8.35</td>
<td>130.6 ± 8.03</td>
<td>91.66 ± 0.97</td>
</tr>
<tr>
<td>Sham-62</td>
<td>154.2 ± 13.5</td>
<td>166.6 ± 11.54</td>
<td>92.08 ± 1.71</td>
</tr>
</tbody>
</table>

Values are means ± SE. Groups defined in METHODS.
that the variability in individual animal performance cannot be explained by number of pores.

Although performance in the Sham-62 and CTX-62R groups did significantly decrease after surgery, this effect was minor in magnitude and short-lived. Repeated-measures one-way ANOVAs conducted across the 5 days of presurgical testing for each group showed no significant differences. However, those conducted on postsurgical testing showed differences in performance across days in the CTX-62R ($P = 0.001$) and Sham-62 groups ($P = 0.013$). Performance tended to improve from day 1 to day 5 during postsurgical testing for these two groups (Fig. 3), suggesting that these animals had forgotten aspects of the task during the 62-day recovery period and recalled it gradually across the postsurgical testing days. In fact, there is no significant difference between performance during the last three sessions of postsurgical testing and the last three sessions of presurgical testing for either of these groups (all $P$ values $> 0.062$). Consequently, the significant drop in postsurgical discriminability in these two groups is likely attributable to the initial and transient decay in performance. Figure 4 illustrates individual animal postsurgical performance for the Sham-62, CTX-62R, CTX-62P, and CTX-7P groups. Although some animals in the CTX-7P and CTX-62P groups modestly improved across postsurgical testing days, performance in others remained steady or worsened. In summary, there was a fair amount of variability in performance of rats without CTs, but, as a whole, their performance is clearly different from, and worse than, that of rats with CTs. Moreover, the performance during the last three sessions of postsurgical testing (before the amiloride test) of rats in the CTX-7P and CTX-62P groups was significantly worse than that observed in the other three groups (all $P$ values $< 0.008$) and was also significantly lower compared with that observed in the same rats during the concomitant presurgical period ($P = 0.005$ and 0.031, respectively).

Group mean difference scores are shown in Fig. 5. Theoretically, the maximum difference score that can be achieved is 0.50, which would represent a change from perfect performance (1.0) to chance performance (0.50). Thus these scores signify surgically induced changes in performance, with larger scores indicating larger effects of surgery. All difference scores were positive, reflecting decreases in performance (to vary-

![Fig. 3. Mean ± SE overall proportion correct for each group on each postsurgical testing day.](http://ajpregu.physiology.org/)

![Fig. 4. Individual animal performance across postsurgical testing days for rats in the Sham-62, CTX-62R, CTX-62P, and CTX-7P groups. Because of the low variability in individual animal performance in the Sham-7 group (see Fig. 3), that group is not included here. Broken line indicates chance performance. R = rat.](http://ajpregu.physiology.org/)
ing degrees) in all groups after surgery. A one-way ANOVA indicated a significant effect of treatment \( F(4,19) = 515.973, P < 0.001 \) on the scores. Tukey pairwise comparisons revealed that scores in the Sham-7, CTX-62R, and Sham-62 groups did not statistically differ but that they did differ from both the CTX-7P and CTX-62P groups (all \( P \) values < 0.029), which, in turn, did not differ from each other.

Amiloride Testing

Performance on the first and second 100 µM amiloride testing sessions was not significantly different in the Sham-7, Sham-62, CTX-62R, or CTX-62P groups. Therefore, the two 100 µM data points for these groups were averaged, and this mean was used as a single 100 µM data point in the following analyses.

Curves were fit to the amiloride testing data (amiloride days) for each rat as well as the means of the Sham-7, Sham-62, and CTX-62R groups (Fig. 6). The resulting parameters of the curve fits for the Sham-62 and CTX-62R groups were compared using independent \( t \)-tests. None of the parameters differed significantly between these two groups. The small sample size of the Sham-7 group during amiloride testing (\( n = 2 \)) precluded meaningful statistical analysis, but the parameters for this group were remarkably similar to those for the Sham-62 and CTX-62R groups (see Fig. 6). The animals with regenerated nerves (CTX-62R) exhibited the same concentration-dependent disruption of performance by amiloride as did the animals with intact nerves (Sham-7 and Sham-62).

Although logistic functions could not be fit to the amiloride data for the CTX-7P and CTX-62P groups (Fig. 7) because of the obvious absence of any sigmoidal shape, a repeated-measures one-way ANOVA across amiloride doses of 0, 0.94, 3, 10, 30, and 100 µM indicated a significant effect of amiloride concentration in the CTX-62P group \( F(5,15) = 7.6, P = 0.001 \). The zero amiloride dose represents mean performance during the control sessions shown in Fig. 7. Paired-sample \( t \)-tests comparing performance at each amiloride concentration to that at the zero amiloride dose for the CTX-62P group showed significant differences (from the 0 dose) at each concentration, as follows: 0.94 µM (\( P = 0.005 \)), 3 µM (\( P = 0.029 \)), 10 µM (\( P = 0.017 \)), 30 µM (\( P = 0.013 \)), and 100 µM (\( P = 0.010 \)). Statistical analyses were precluded in the CTX-7P group because of the small sample size during amiloride testing (\( n = 2 \)). However, when the animals in the CTX-7P group were
combined with those in the CTX-62P group and the above statistical tests were conducted, the significance of the outcomes remained the same.

Water Control Test

None of the rats performed better than chance during the water control test, indicating that all animals were responding to the chemical nature of the stimuli throughout testing rather than any unidentified extraneous cues.

DISCUSSION

The results presented here show that transection of the CT impairs the ability of rats to discriminate between NaCl and KCl, replicating previous work (62, 65, 66). More importantly, we were able to demonstrate that regeneration is the causal factor in recovery of function after CTX. For rats in which regeneration was prevented, time alone, which could allow for the strengthening of remaining ability and/or reorganization of central neural circuits, was not sufficient to result in any significant functional recovery. In fact, performance on the salt discrimination task among CTX rats (regeneration prevented) receiving a 62-day recovery period was not different from those receiving a much shorter 7-day recovery period. Thus these results importantly extend the earlier work of St. John et al. (66), which showed that salt discrimination returned to normal upon CT regeneration, in demonstrating that reappearance of taste buds is a necessary condition for functional recovery in this task.

When performance was examined on an individual animal basis, CTX did not appear to have any systematic effects with regard to stimulus or concentration. In the CTX-7P and CTX-62P groups, some rats displayed more errors on NaCl trials relative to KCl, and in other rats the converse was true. Some rats improved performance as a function of concentration of one of the salts, and other rats exhibited declines in performance as the concentration was raised. This variability is not entirely unexpected because CTX is known to affect the taste sensibility of both salts (e.g., see Refs. 24, 62, 64–66). Moreover, when discrimination becomes difficult, the idiosyncratic response biases of individual subjects are more likely to be expressed.

Examination of individual animal performance also showed that CTX (CTX-7P and CTX-62P) rats were impaired to various degrees, with some scoring above chance while others did not. This variability in individual animal salt discrimination performance is similar to that observed in other studies involving identical (65) and different procedures (66). In the present study, we found no significant correlation between the number of taste pores and overall performance in the animals with transected CTs. Thus the variability in these CTX groups cannot be easily explained by the number of taste buds. Similarly, no significant correlation was observed between overall performance and number of taste pores in animals with regenerated or intact nerves. Therefore, we can state that rats with high numbers of pores performed well on this task, and those with few pores performed poorly, but this was more likely due to the presence or absence of the CT, so we cannot strictly say that number of pores predicted performance. In general, this replicates the findings of St. John et al. (66), but, unlike the prior study, we did not find evidence for a gradual relationship between number of regenerated pores and performance. However, if we had used a surgical procedure or survival times that would have produced rats with more intermediate numbers of taste buds, perhaps a more progressive relationship would have been revealed.

It is interesting to note that human psychophysical studies have found correlations between anterior tongue taste pores and the perceived intensity of certain taste compounds, most notably 6-n-propylthiouracil (PROP; see Refs. 4, 42, 52). Although in the present study we found no significant correlation between performance and number of taste pores among rats with intact or regenerated nerves, the task that we used was markedly different than those used in the human psychophysical work. For example, we used a discrimination
task, whereas suprathreshold magnitude estimation is often used with humans. Also, the present study focused on two taste compounds, whereas human research often involves a wider array of tastants, most notably PROP, sucrose, NaCl, citric acid, and quinine (4, 42, 52). Thus it remains possible that a relationship exists between the number of anterior tongue taste buds and the perceived intensity of suprathreshold concentrations of particular taste stimuli in the rat. Moreover, it has been shown that return of localized sensitivity to citric acid in a denervated region of the anterior tongue relates to the reappearance of anterior tongue taste pores in humans sustaining CT injury followed by repair (70).

We did obtain certain results that differ slightly from those of St. John et al. (66). In the previous study, the number of taste pores in rats with regenerated nerves reached an asymptote of \( \frac{2}{3} \) the number in control animals at 42, 56, and 70 days after CTX. In the present study, we found that the number of taste pores in the animals with regenerated nerves (CTX-62R) reached \( \approx 78\% \) of the control animals in the Sham-62 group and 83\% of the control animals in the Sham-7 group. Because our animals in the long-term groups underwent 1 wk of postsurgical testing followed by 3 wk of amiloride testing beginning 62 days after surgery, they were not actually perfused until \( \approx 90 \) days after surgery. The longest survival time in the study by St. John et al. was 70 days. It is possible that the additional 20 days of survival allowed for the regeneration of a greater number of taste buds in our study, although the asymptote in taste bud regeneration from days 42–70 shown by St. John et al. would seem to argue against this. In any event, the mean number of taste pores was less in the CTX-62R group relative to controls, and the failure to find significance more likely relates to the statistical power of the tests due to the small sample size given the variability of the effect. In a recent study just completed in our laboratory, we found that rats with regenerated CTs perfused 118 days after surgery had only 62–70\% of the taste pores observed in their sham-operated counterparts (36).

Although the postsurgical performance of the rats with regenerated nerves was statistically different from their presurgical performance, this is probably best explained by a slightly degraded memory of the task, due to the long recovery period. This is supported by the fact that the animals with intact nerves that also received the 62-day recovery period exhibited an almost identical decline in performance after surgery. The suggestion that the rats had, to a small degree, forgotten the task during the extended recovery period is also supported by the fact that their performance improved across postsurgical testing sessions, as well as the fact that their mean performance during the last 3 days of postsurgical testing was not significantly different from that during the corresponding 3 days of presurgical testing. Performance of four CTX rats (rats 2, 10, 19, and 17) also tended to improve across postsurgical testing days. This may indicate that these rats were able to use the signal that remained after CTX better than others, which was then strengthened as a result. The performance of these animals was clearly worse than the performance of those with intact nerves, however, suggesting that different mechanisms were underlying the discrimination in rats with and rats without CTS.

Rats with regenerated nerves exhibited the same degree of sensitivity to amiloride as rats with intact nerves, and their performance was disrupted in the same concentration-dependent manner. These results complement those of earlier studies, which have shown that electrophysiological responses of regenerated CTS are suppressed by amiloride (29, 44). Our data extend these prior findings by showing that the amiloride-induced disruption in performance in rats with regenerated CTS is dose dependent and thus imply that amiloride-sensitive channels in taste receptor cells innervated by the regenerated CT are contributing to the recovered function. In fact, based on our behavioral assay, the functional status of the amiloride-sensitive transduction pathway (see Refs. 8, 15, 45, 69) in regenerated taste buds appears to be completely normal. Our results from amiloride testing in the Sham-7, Sham-62, and CTX-62R groups parallel those of Spector et al. (63), who found that amiloride disrupts salt discrimination performance in a dose-dependent manner. Additionally, when we examined performance by the type of salt delivered on each trial, we found that amiloride had its greatest performance-disruptive effects on NaCl, results that also parallel those of Spector et al. (63). That is, amiloride disrupted performance on KCl trials only to a minor degree, whereas the overall proportion of correct responses to NaCl dropped to below chance at high amiloride doses (cf. Fig. 3 of Ref. 63). In other words, on NaCl trials in which the NaCl was mixed with high concentrations of amiloride, rats pressed the KCl-associated lever more than the NaCl-associated lever, suggesting that they actually perceived the amiloride-adulterated NaCl as more similar to KCl than to NaCl.

Amiloride did not appear to have this same concentration-dependent disruption of performance in the CTX-7P and CTX-62P groups. This lack of a dose-dependent function may result from the fact that performance on control days was already approaching the chance level, which is theoretically the lowest level of performance possible. Therefore, the range between the already low performance on control days and chance performance may not have been large enough for a clear concentration-dependent function to emerge. Although we were unable to fit curves to the amiloride data for the CTX-7P and CTX-62P groups, the analyses show that performance, even though already significantly impaired, was further degraded by amiloride at all concentrations tested. These findings indicate that some amiloride-sensitive taste receptor cells may be innervated by gustatory nerves other than the CT. This suggestion is complemented by recent findings of Sol-lars and Hill (59), who demonstrated that amiloride suppresses the NaCl responsiveness of the GSP, as well as those of Roitman and Bernstein (54), who demon-
strated that amiloride treatment decreased the expression of a sodium appetite to a greater extent than did CTX alone.

According to the measures and results examined here, the regenerated CT appears to be as functional as the intact nerve when assessed behaviorally. Moreover, nerve transection-induced reorganizational events or strengthening of remaining signals in the gustatory system, if occurring, do not appear to compensate for the absence of CT input, at least not under the conditions tested in our study. Definitive conclusions regarding the functionality of regenerated gustatory nerves in general or the possibility of compensatory changes in the nervous system should not be made solely on the basis of this study, however. The discrimination task used here examines only one aspect of taste-guided behavior with a limited stimulus array. Moreover, only one taste nerve, the CT, which innervates only 15% of the total taste receptors, was investigated. It would be instructive for researchers to assess the functional capacity of the regenerated CT with other tests, including those that measure sensitivity, hedonics, and cephalic phase responsiveness.

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