Effect of Benzodiazepines within the PBN on Taste Guided Licking Behavior

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A research thesis submitted in partial completion of PSY452 senior thesis, at Wofford College,

Spring 2014
CDP INCREASES PALATABILITY OF TASTANTS

Abstract

Research shows that benzodiazepines, specifically chlordiazepoxide (CDP), increases palatability through a GABA_A agonistic effect. The parabrachial nucleus (PBN) in the hindbrain may represent a site of action. The purpose of this study was to examine the effects of directly administered CDP into the PBN on the palatability of aversive tastants. Bilateral cannulae were inserted into the PBN of naïve male Sprague-Dawley rats. Subjects received microinjections of CDP or artificial cerebrospinal fluid (aCSF) and were exposed to brief access trials of varying concentrations of sodium chloride, quinine, and citric acid. Results showed that rats injected with CDP displayed more licks at intermediate concentrations. Our results suggest the endogenous presence of GABA receptors in the PBN that may be able to modify the hedonic nature of tastants.
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Taste recognition is important in regards to survival mechanisms. When deciding whether or not to ingest a substance, one must remember the post-ingestive effects related to the gustatory stimulus from previous encounters (Saggu & Lundy, 2008). For instance, a taste that has been associated with internal malaise would decrease likelihood of intake. On the other hand, a taste that has been associated with nutritive gastrointestinal consequences would increase intake probability (Fanselow & Birk, 1982). Thus, the change in hedonic value underlies the learned gustatory behavioral response for future food consumption (Saggu & Lundy, 2008).

The rat parabrachial nucleus (PBN), a second-order relay of the gustatory system, may be the main integrator and modulator of necessary input to change future feeding behavior (Reilly & Trifunovic, 2000; Karimnamazi, Travers, & Travers, 2002). As the first relay station of the taste pathway, the nucleus of the solitary tract (NST) sends both taste and visceral input to the PBN (Herbert, Moga, & Saper, 1990). The gustatory input is concentrated in the medial region of the PBN (mPBN) while the gastric component is more prominent in the lateral section of this nucleus (lPBN) (Reilly & Trifunovic, 2000). The PBN also receives descending projections from forebrain regions such as the central nucleus of the amygdala (CeA), the lateral hypothalamus (LH), the bed nucleus of the stria terminalis (BNST), and the gustatory cortex (GC) (Lei et al., 2008; Huang, Yan & Kang, 2003; Lundy Jr. and Norgren, 2004; Zhang, Kang & Lundy, 2011). Hence, a myriad of projections and circuits to the PBN assist the modification of incoming taste signals to form either aversive or preferred behavioral responses.

The PBN is also the main structure responsible for modulation of taste preferences and aversions. With respect to conditioned flavor preferences (CFP), procedures typically consist of pairing a flavored solution with positive post-ingestive effects. The tastant represents the
conditioned stimulus (CS) and the visceral feedback from intragastric (IG) nutrient infusions is regarded as the unconditioned stimulus (US) (Uematsu, Tsurugizawa, Kondoh, and Torii, 2009). Since appetitive taste formation is conditioned through visceral effects, the IPBN has been the target in many taste preference studies. Findings have indicated that rats with IPBN lesions are unable to associate the positively reinforcing consequences of a nutritional substance with its respective gustatory stimulus (Reilly and Trifunovic, 2000; Zafra, Simón, and Puerto, 2002). It has also been demonstrated that electrical stimulation of the IPBN can modify the hedonic value of flavors to become more palatable (Simon, Garcia, Zafra, Monlina, Puerto, 2007).

By contrast, rats learn to avoid intake of a substance in conditioned taste aversion (CTA) paradigms. Taste aversions are established by associating a taste stimulus (CS) with the injected toxic drug consequences of internal malaise (US) (Sclafani, Azzara, Touzani, Grigson, and Norgren, 2001). Rats with excitotoxic, ibotenic acid lesions of the PBN have displayed impaired aversive behavior when paired with intraparietal injections of lithium chloride (LiCl) (Yamamoto, Fujimoto, Shimura, and Sakai, 1995). More specifically, mPBN lesioned rats demonstrate an association deficit between taste and visceral input (Aguero, Gallo, Arnedo, Molina, and Puerto, 1997), while IPBN lesioned rats lack the ability to detect US-related gut-feedback (Reilly and Trifunovic, 2000).

Along with hedonic changes, another means in modification of PBN taste-guided behavior is the neurotransmitter GABA. Both taste and visceral input into the PBN is influenced by the inhibitory function of GABA, primarily through GABA_A receptors (Kobashi and Bradley, 1998). Moreover, it has been implied that the connections between descending corticofugal terminals and the taste nuclei of the PBN are mediated by GABAergic innervations (Saggu and Lundy, 2008). It has also been demonstrated that a more enhanced effect of GABA can be
produced through administration of benzodiazepines, an anxiolytic drug. By particularly acting on the GABA<sub>A</sub> receptor composite of the PBN circuit, benzodiazepines such as midazolam and chlordiazepoxide (CDP) can increase oromotor responses to preferred tastes as well as reduce avoidance reactions to aversive tastes (Berridge and Treit, 1986; Söderpalm and Berridge, 2000).

In distinguishing benzodiazepines-mediated effects on the PBN, Pittman et al. (2012) investigated whether CDP modified the perceived intensity or the hedonic evaluation of taste stimuli. Using microstructure analysis of licking behavior, they assessed the influence of systemically applied CDP on consummatory behavior across three concentrations of the basic taste qualities during a long-term 1-h test sessions. Findings indicated that due to a reduction in pause duration, motivation for intake combined with taste-mediated responses to most stimuli resulted in increased consumption for both appetitive as well as aversive tastants. In other words, CDP increased the positive hedonic evaluation of the stimuli affecting the perceived intensity.

This experiment expands upon that research study by examining the effects of CDP on taste-guided behavior during a brief-access test session. Through brief-access testing, we recorded licking to different concentrations of three aversive tastants (NaCl, citric acid, and quinine) with and without the administration of CDP directly within the PBN. Due to previous findings on benzodiazepines use, we hypothesized that intraPBN CDP injections would increase licking behavior to tastants. We believe that CDP will influence licking of concentrations of aversive tastants that do not elicit maximal licking (little taste influence on behavior) or minimal licking (strong aversive taste signals). We predict no effect on the latency until the first lick, as CDP is active on taste signals rather than motivation to lick.
Method

Subjects
Nineteen naïve, male Sprague-Dawley rats (Charles River Laboratory Raleigh NC) were single housed at the beginning of training in transparent plastic cages within a climate-controlled room with a 12:12-h light:dark cycle (lights off 1200). Rats had free access to rodent chow and deionized water unless otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee of Wofford College.

Apparatus

Short term. All testing procedures were performed in the MS-160 gustatory apparatus (DiLog Instruments, Tallahassee, FL). The MS-160 allowed the controlled presentation of up to 16 chemical stimuli while recording licking behavior of the animal at 1-ms resolution. The sliding stimulus rack and motor-controlled lever shutter covering the access port allows for programmed presentations of taste stimuli for controlled durations, pause periods, and interstimulus intervals (Smith 2001). The MS-160 was housed within an acoustic isolation chamber equipped with a white noise generator. Intake and exhaust fans were located on opposing walls of the chamber to sustain a continuous flow of air along the longitudinal axis of the stimulus delivery system. An 8-watt light illuminated the housing chamber.

Long term. CTA training was performed in the AC-108 (DiLog Instruments, Tallahassee, FL) using standard rat cages, without bedding, equipped with a single lickometer device to record contact with licking spout at a 1-ms resolution.

Drug and Reagents
All chemical tastants were mixed daily from reagent grade chemicals dissolved in deionized water and presented at room temperature. Stimuli and concentrations consisted of citric acid
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(citric acid, 0.008, 0.015, 0.03, and 0.06 M), sodium chloride (NaCl, 0.125, 0.25, 0.5, and 1 M), sucrose (0.2 M); were obtained from EMD Millipore Chemicals, (Gibbstown, NJ). Quinine hydrochloride (0.01, 0.05, 0.2, and 0.8 mM) was obtained from MP Biomedicals (Solon, OH). Lithium chloride (LiCl, 0.3 M; Sigma-Aldrich, St. Louis, MO) was used as the unconditioned stimulus evoking gastric malaise in the conditioned taste aversion tests. CDP (33.6 μg) was dissolved in artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Hilliston, MA) and aliquots were frozen until the day of testing.

Procedure

Training. All rats were put on a 23-h water restriction schedule during the training period prior to testing. Rats received three days of training in the MS-160. The first day rats received a deionized water stimulus. The second and third day of training rats received 0.2 M sucrose, 0.1 M NaCl, 0.0005 M quinine, 0.0008 M citric acid, and water. Stimuli were presented using 30-s stimulus durations and 10-s interstimulus intervals. After each testing session a 15-min wait period was implemented followed by 15-min water access in the home cage.

Surgery. Following the completion of training, rats (300-330g at time of surgery) were anesthetized with an initial ketamine (100 mg/ml) and xylazine (10 mg/ml) mixture (0.35 ml) and atropine (0.25 ml) with supplemental ketamine/xylazine mixture (0.15 ml) as needed. Once secured in the stereotaxic apparatus (David Kopf Instruments; Tujunga, citric acid), 25-ga guide cannulae (PlasticsOne; Roanoke, VA) were implanted bilaterally 1mm dorsal to the PBN. Coordinates were +0.7 mm anterior, ± 2.0 mm lateral, −7.7 mm dorsal and at a 20 degree angle relative to lambda. Subjects were allowed a minimum of three days recovery before the start of testing and cannulae were agitated daily.
Drug administration. Microinjections were made using a 33ga microinjector that extended 1mm below the guide cannulae. Injectors were connected to a 1 μg Hamilton syringe by polyethylene tubing (PE20). A pump (KD Scientific) was used to deliver 4μL of aCSF or CDP at a rate of 2μL/min for 2 minutes; the injectors were left in place for another 2-min after the injections to allow for diffusion. Rats were then placed into their home cage without access to food or water for 15-min before they were placed in the MS-160 chambers for a test session.

Testing. Stimulus delivery parameters were 15-s stimulus trials with 30-s wait period for the first lick for the duration of testing procedure. Tastant delivery order was in pseudorandomized blocks of NaCl, critic acid, and quinine, with one water trial included in each tastant block. Each daily test session consisted of three blocks of each concentration of the tastant. After the completion of all test sessions, a 30-min wait period was implemented followed by 15-min-water access for all the rats. Rats were tested at 48-hr intervals with 1-hr water access on non-testing days.

Conditioned Taste Aversion. Following the completion of the brief-access testing, rats underwent conditioned taste aversion (CTA) training. Using the AC-108 long-term testing chamber, rats were given 60-min access to sucrose (0.2 M). Immediately after the completion of the first training session, rats were given a LiCl injection (0.3 M i.p. 1 ml/kg body weight). The following day, animals were retested for the expression of a CTA. A 25% decrease, from day one, in licks was used at the criterion for a conditioned taste aversion. One animal was excluded from the analysis on the basis of the CTA criterion.

Histology. For histological verification, all animals were deeply anesthetized with ketamine and xylazine mixture (0.45mL). Rats were microinjected bilaterally with 4μL with Chicago Sky Blue 6B (Sigma-Aldrich) to measure estimated drug diffusion. The rats were then perfused
transcardially with saline, followed by 10% formalin. Brains were extracted and stored in formalin and then transferred into a 10% formalin/10% (w/w) sucrose solution until sectioned.

**Data analysis.** The latency to the first lick, the total number of licks per 15-s stimulus duration, and interlick interval (ILI) were recorded for each trial. The mean lick response was calculated for each concentration of the tastant over the three blocks for each test session. Mean licks per tastant concentration were converted into a lick ratio by dividing the licks per tastant concentration by the average licks to water in a given test session to normalize individual differences in thirst motivation. Repeated-measures ANOVA analyses were performed for the lick, ILI and latency trials. Post-hoc t-tests were performed to examine significant differences at specific tastant concentrations between drug and control conditions.

**Results**

**Licks**

As hypothesized, CDP significantly increased licking as measured by lick ratio for all three tastants. As shown in Figure 1, there was a significant main effect of CDP on the lick ratio for NaCl, $F(1, 45) = 4.91, p = .03$. A post-hoc paired t-tests revealed that CDP significantly increased the mean lick ratio at the 0.5 M NaCl concentration, $p = .03$.

For citric acid solutions, there was a significant main effect of drug on the mean lick ratio, $F(1, 44) = 5.956, p = .019$. As shown in Figure 2, there was a significant interaction between CDP and tastant concentration on mean lick ratio, $F(3, 132) = 3.451, p = .019$. Post-hoc paired t-tests revealed that CDP significantly increased the mean lick ratio at both the 0.03 M ($p = .02$) and 0.06 M ($p < .01$) citric acid concentrations.

As shown in Figure 3, there was a significant main effect of CDP on the mean lick ratio for quinine, $F(1, 50) = 4.109, p = .048$. There was no significant interaction between drug and
tastant concentration. Post-hoc paired t-tests revealed that CDP significantly increased the mean lick ratio at the 0.2 mM quinine concentration, $p = .02$.

**Interlick Interval (ILI)**

Interlick Intervals (ILIs) represent the mean delay (ms) between licks within a trial. For water and normally accepted taste stimuli, ILIs are consistently within a range of 150-200 ms due to a central motor pattern generator controlling the licking behavioral response. However, as tastants become aversive there are increases in the duration of ILIs. The presence of CDP did not significantly affect the ILIs compared to aCSF controls. As shown in Figure 4, there was a significant main effect of NaCl concentration on ILI, $F(4, 76) = 10.488$, $p < .001$. Post-hoc t-tests revealed that ILI was significantly higher for 1.0 M NaCl compared to all other stimuli in the NaCl block ($ps < .05$).

As seen in Figure 5, there was a significant main effect of citric acid tastant concentration on ILI, $F(4, 76) = 15.33$, $p < .001$. Post-hoc analyses revealed that ILI for water was significantly less than 0.015 M, 0.03 M, and 0.06 M concentrations of citric acid, ($ps < .05$). The ILI of 0.0075 M citric acid, $p = .005$, and 0.03 M citric acid, $p = .017$, were significantly less than ILI for 0.06 M citric acid.

As shown in Figure 6, there was a significant main effect of quinine tastant concentration on ILI, $F(4, 76) = 22.72$, $p < .001$. Post-hoc analyses revealed ILI for 0.2 mM quinine was greater than for water, $p = .043$, and .01 mM quinine, $p = .02$, and less than for .8 mM quinine, $p = .001$. ILI for .8 mM quinine was greater than for water, $p = .001$, and .01 mM quinine, $p < .001$, and less than for .05 mM quinine, $p = .002$. 
Latency

Latency refers to the mean time (s) that passed before the first lick of each trial. Whereas CDP did not affect latency, there was a significant effect of tastant concentration. For NaCl solutions, there was a significant main effect of tastant concentration on latency, $F(4, 76) = 3.876, p = .006$. As shown in Figure 7, post-hoc analyses revealed that latency for 1.0M NaCl was significantly greater than for 0.125 M NaCl, $p = .035$, and 0.5 M NaCl, $p = .014$.

For citric acid solutions, there was a significant main effect of tastant concentration on latency, $F(4, 76) = 23.781, p < .001$. As shown in Figure 8, post-hoc analyses revealed that latency for water was significantly less than all citric acid concentrations, ($p$s < .001). Latency for 0.0075 M citric acid was significantly less than 0.015 M citric acid, $p < .001$.

For quinine, there was a significant main effect of concentration on latency, $F(4, 76) = 3.105, p = .02$. As shown in Figure 9, post-hoc analyses revealed that the latency associated with 0.01 mM quinine was significantly less than that of 0.05 mM quinine, $p = .05$, and significantly greater than for water, $p < .01$. Latency for 0.05 mM quinine was significantly less than 0.8 mM quinine, $p = .02$, and greater than for water, $p < .001$.

Conditioned Taste Aversion

As shown in Figure 10, we were able to condition a taste aversion in each rat, as each subject licked at least 75% less than the previous day after being injected with LiCl. Subject AV12 did not form a CTA that was accepted by our criteria and was dropped from the experiment.

Discussion

The purpose of this experiment was to examine the effect of administering CDP directly to the PBN on the feeding behavior of salty, sour, and bitter aversive tastants. Results revealed
that subjects that were administered CDP exhibited more licks compared to subjects that were administered a control injection. The experiment also showed that the concentration of tastant had a significant effect on the interlick interval and latency of licking with no drug effect for either of these variables.

**Licks**

Results in this experiment supported the hypothesis that administration of CDP into the parabrachial nucleus would increase the number of licks emitted for each tastant. Across all tastants, subjects that received CDP exhibited a higher lick ratio compared to subjects that were administered a control solution. For lower concentrations of tastants, there were no significant differences in licking ratios. This is presumably due to lower concentrations of the tastants not having a significant appetitive or aversive effect different from water. In other words, for low solution concentrations, there was not a sufficient taste component to the solution in order for the drugs to affect palatability and increase licking. However, as the concentration of tastant rose, lick rate for subjects administered CDP was significantly greater than that of subjects administered a control injection. For all three tastants subjects given CDP emitted a higher number of licks as the concentration increased. However, this effect was not seen at extremely high levels of concentration, which were presumably so aversive that the negative taste overrode the drug effect. Except for citric acid, there was no significant difference in lick ratios at high concentrations. Thus, CDP created a “U” shaped dose-response curve, with significant differences occurring at concentrations falling within the middle of the curve.

**Interlick Interval**

Results revealed a significant main effect of tastant concentration on interlick interval. This was seen across all three tastants: salty, sour, and bitter. As tastant concentration increased,
interlick interval also increased. This may be due to rat orofacial behaviors associated with aversive tastants. Rats display marked orofacial behaviors, such as gaping, face washing, and headshakes after tasting aversive stimuli. Therefore, interlick interval would be higher for more aversive tastants since rats may spend more time displaying these behaviors, interrupting their normal licking pattern. Contrary to licking behavior, this means that CDP did not modify the hedonic value of tastants relative to ILI. Thirty-second trials may not have been long enough to accurately measure ILI. These insignificant results may be because ILI is an inappropriate measure of gauging appetitive/aversive behavior in brief-access access trials.

**Latency**

Contrary to our initial hypothesis, results revealed a significant main effect of tastant concentration on latency. The main effect was observed across all tastants: salty, sour, and bitter. Subjects took longer to begin initiating licks as the concentration increased. This may be due to rats’ enhanced olfactory senses. Despite taken precautions, such as intake and exhaust fans, rats may have been able to smell the high concentrations of each tastant. Thus, subjects were less likely to approach the solution. The significant main effect of concentration may have also been contributed to the methodology used in the experiment. Although there was a significant statistical effect of tastant concentration there was no practical delay in approaching the licking spout. All latencies were under 10-s of the 30-s wait period.

**Role of the PBN in Taste Evaluation**

These results implicate the involvement of the PBN in modifying the hedonic quality of tastants. Our findings are supported by several lesion studies, which also designate the PBN as an important brain site in the evaluation of hedonic stimuli. In an experiment conducted by Reilly and Trifunovic (2000), the effects of bilateral ibotenic acid lesions of the IPBN on intake
of four basic taste stimuli (sucrose, NaCl, citric acid, and quinine HCl) were examined. The findings demonstrated that rats with lesions to the lPBN resulted in a concentration-dependent consumption of sucrose, a mild disturbance of sodium chloride consumption, and no effect on citric acid and quinine intake. In other words, damage to the lPBN altered the hedonic quality of the appetitive tastants, sucrose and low concentrations of sodium chloride, but not for the aversive tastants, citric acid and quinine. This non-impact of lesions on citric acid and quinine suggests that responses to these aversive stimuli are controlled more by the gustatory-centered mPBN instead of the viscerosensory neurons of the lPBN.

Reilly and Trifunovic’s (2000) results were supported by further findings examining lPBN lesion effects’ on aversive and appetitive gustatory conditioning. In another study, Reilly and Trifunovic (2000) examined the performance of parabrachial nucleus-lesioned subjects in learned preference and aversion tasks. Rats were trained to exhibit conditioned taste aversions and conditioned taste preferences. Results showed that subjects with lesions to the lPBN failed to demonstrate both conditioned taste aversions and preferences. Taking together the results from the two studies, Reilly and Trifonovic posit that lesions to the lPBN reduce sensitivity to gastrointestinal feedback, preventing modification in taste learning mechanisms (Reilly & Trifunovic 2000).

The mPBN, on the other hand, has also been shown to be necessary for changes in taste palatability. Sakai and Yamamoto (1998) investigated the role of both the mPBN and the lPBN in acquisition and retention of CTA’s. By establishing electrolytic lesions to these designated areas both before and after conditioning procedures, they were able to decipher the functional roles of the distinctive parts of the PBN. While the lPBN-lesioned rats only demonstrated impaired acquisition of learning, mPBN-lesioned rats failed in acquisition and retention of CTA
formation. In other words, these results indicated that both the lPBN and mPBN are crucial for acquisition of taste aversion learning, but only the mPBN is necessary for retention purposes. Therefore, the PBN as a whole plays an important role in shifts in palatability and different processes on taste memory formation.

This ability of the PBN to modify the hedonic quality of tastants may be due to connections between the PBN and other forebrain substrates. In a study conducted by Lundy Jr. and Norgren (2004), the effect of lateral hypothalamus and gustatory cortex input as well as the convergence of these sites, along with the central nucleus of the amygdala, on PBN gustatory processing was evaluated. Responses to the four sapid stimuli were recorded in 70 PBN neurons before, during and after electrical stimulation of the forebrain structures. The findings demonstrated that each forebrain site had modulated PBN responses; however, the stimulation of the GC (67%) and the CeA (73%) resulted in a greater influence on neuronal taste-responses than that of the LH (48%). Also, in regards to the convergence of centrifugal input, integration of the forebrain afferents were detected in most of the PBN neurons tested (70%). Hence, the descending input from these structures helps in modifying the PBN taste-evoked behavior.

Conclusion

In summary, evidence from this study and past experiments has heavily implicated the PBN and the role it plays in modulating the hedonic quality of tastants. Stimulation of the PBN has been shown to enhance the palatability of taste stimuli, whereas lesions to this area have been shown to disrupt conditioned taste aversions and taste preferences. This effect might be due to the interconnections between the PBN and forebrain areas, as shown by the ability of these substrates to modulate the activity of parabrachial nuclei responding.
As previously stated, such interconnections between corticofugal input and the PBN are mediated by GABAergic innervations, which may be responsible for the changes in hedonic value shown in this experiment. By directly acting on GABA$_A$ receptors in the PBN, benzodiazepines are capable of affecting taste palatability with enhancement of GABA’s natural inhibitory effect. Since the intraPBN administration of CDP affected responses in taste palatability, it is most likely that the neurotransmitter GABA was responsible for this modulation of afferent taste signals. Therefore, GABA has a specific and critical role in the PBN with respect to taste-mediated changes.

These findings are important because, by finding a way to modulate the hedonic quality of food, researchers may be able to combat the growing obesity epidemic throughout the world. Obesity has been a growing cause for national concern due to the amount of deaths associated with obesity and increased cost in healthcare. By establishing a technique in which a drug can alter the palatability of food, researchers may be able to create a drug that diminishes the appetitive aspects of unhealthy foods and enhances the hedonic quality of more healthy food. This research is still in its infancy; therefore, it is important to continue research in hopes of finding an efficacious and safe way to combat obesity.
References


Figure 1. Relationship between lick ratio and NaCl concentration. Error bars represent standard error of the mean.
Figure 2. Relationship between lick ratio and citric acid concentration. Error bars represent standard error of the mean.
Figure 3. Relationship between lick ratio and quinine concentration. Error bars represent standard error of the mean.
Figure 4. Relationship between average inter-lick interval (ILI) and NaCl concentration. Error bars represent standard error of the mean.
Figure 5. Relationship between average ILI and citric acid concentration. Error bars represent standard error of the mean.
Figure 6. Relationship between average ILI and quinine concentration. Error bars represent standard error of the mean.
Figure 7. Relationship between average latency and NaCl concentration. Error bars represent standard error of the mean.
Figure 8. Relationship between average latency and citric acid concentration. Error bars represent standard error of the mean.
Figure 9. Relationship between average latency and quinine concentration. Error bars represent standard error of the mean.
Figure 10. Licks for session testing conditioned taste aversion development. Values represent licks one day following development of CTA as percentage of previous day's licks.