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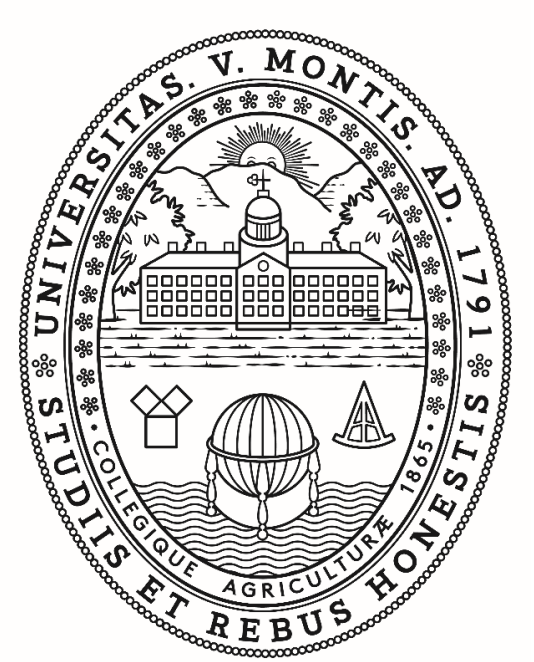

Department of Biology Honors Thesis
The University of Vermont

Examining Committee:

Lawrence Rudiger, Ph.D., Committee Chair
Department of Psychological Science

Eugene Delay, Ph.D., Thesis Advisor
Department of Biology, Neuroscience Program

Amanda Yonan, Ph.D.
Department of Biology

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Abstract

Chemotherapy is a common cancer treatment, yet it has many severe side effects including altered taste. Patients report that salt taste is most affected by chemotherapy. The salt taste transduction system has yet to be fully elucidated. Type I taste cells are thought to be responsible in part for salt taste. The goal of this study was to determine how cyclophosphamide (CYP), a common chemotherapeutic agent, affects salt taste in mice. This involved two experiments. The first experiment examined how an induced conditioned taste aversion (CTA) to NaCl (salt) would change following CYP treatment. The second used a brief access test to observe how NaCl preference changed before and after either a single dose or multiple dose CYP treatment. We hypothesized that CYP would affect Type I taste cells leading to changes in salt preference, that CYP would reduce salt aversion, and that multiple doses would affect multiple salt taste cell types leading to more significant changes in salt preference. Our results demonstrated that after treatment, CYP mice had higher NaCl lick rates than control mice. This occurred in two phases, initially around day 8 and again around day 18. CTA mice maintained an aversion to NaCl following treatment, indicating a pathway protected from CYP disturbance. A single CYP injection and multiple CYP injections had the same effects on mice, indicating that this methodology is not useful in disturbing multiple salt taste cell populations. These data support that there are at least two salt taste transduction pathways in mice.

Chapter I: General Overview

Introduction

Chemotherapy is one of the most widely used cancer treatments. However, it can have many severe side effects that impact patients' quality of life. One significant chemotherapy-related side effect is altered taste. In a study of 518 patients undergoing chemotherapy, taste changes were self-reported by 67% of the patients, with 56% of patients experiencing oral problems and 22% experiencing appetite loss. Of the 67% of patients who reported taste changes, 41% reported that salt taste was affected, while sweet, bitter, and sour were less affected (36%, 24%, and 21% respectively) (Bernhardson, Tishelman, & Rutqvist, 2008). Salt, specifically NaCl, is one of the most widely used flavoring agents in many cultures. The addition of salt is not only used as a flavor enhancer itself, but can also be used to modify other tastes, such as masking bitter flavor and enhancing the perceived sweetness of a meal (Breslin & Beauchamp, 1997). Salt also increases the intensity of umami taste and of weak citric acid but decreases the intensity of lactic acid and strong citric acid (Kawasaki, Sekizaki, Hirota, Sekine-Hayakawa, & Nonaka, 2016). Some degree of the weight loss that affects 22% of chemotherapy patients is likely attributable to appetite loss, which can further be attributed to taste changes (Kiss, Isenring, Gough, & Krishnasamy, 2014). Additionally, quality of life is greatly reduced if patients no longer enjoy eating since it serves not only a nutritional but also a social role in daily life (Bromley, 2000). Taste changes are the second most bothersome effect of chemotherapy reported in patients, with hair loss being first (Lindley et al., 1999). The first step in mitigating these taste changes is to further understand the mechanism in which chemotherapy affects salt taste receptor cells, which was the goal of the present study.

Background on taste

The mammalian taste system is a complex set of receptors and pathways that has yet to be fully understood. Most mammals are able to sense five basic tastes: salty, sweet, bitter, sour, and umami. Between 2000 and 5000 onion-shaped epithelial chemosensory organs known as taste buds are located in the human oral cavity. These taste buds are distributed on the tongue, palate, epiglottis, pharynx, and larynx. The taste buds on the tongue are located in three distinct regions which are characterized by three different papillae, including fungiform, circumvallate, and foliate. Fungiform papillae are located on the anterior of the tongue, circumvallate papilla are located on the posterior of the tongue, and foliate are on the lateral sides of the tongue. Fungiform papillae are innervated by the chorda tympani nerve and circumvallate papillae are innervated by the glossopharyngeal nerve. Foliate papillae are innervated by branches from both the glossopharyngeal and the chorda tympani nerves. Taste buds located in the palate are innervated by the greater superficial petrosal nerve, which is a branch of the facial nerve (Roper, 2013).

The three types of taste sensory cells are Type I, Type II, and Type III cells. (Yarmolinsky, Zuker, & Ryba, 2009). A fourth type of taste cell, Type IV cells, are undifferentiated cells which will mature to become either Type I, II, or III cells. Type I cells are defined by their electron-dense cytoplasm and elongate, pleomorphic nuclei and have glial-like function. These cells are characterized by lamellar processes that wrap the other taste cells. They also act as insulators by producing ecto-ATPase, which degrades the chemical signals of the other taste cells. Type II cells are defined by their expression of G protein-coupled receptors. While these cells do not possess typical synapses, it is now known that they secrete ATP which acts as a neurotransmitter to sensory afferent fibers. Type III cells, also known as presynaptic

cells, possess synapses and act as an intermediate between Type I and Type II cells. These cells secrete serotonin, norepinephrine, and GABA (Roper, 2013). Taste cells all have different lifespans, with Type II cells having a half-life of eight days and Type III cells having a half-life of 22 days. Type I cells, the cells of greatest interest in the present study, show two separate populations which have very different longevities. Three-fourths of the Type I cells have a half-life of eight days, while the remainder of the Type I cells live for 24 days. It is predicted that the cells with a 24-day lifespan are likely immature, undifferentiated taste cells, while the cells with an eight-day lifespan are mature Type I cells (Perea-Martinez, Nagai, & Chaudhari, 2013).

Each cell type has a specific detection and transduction stimuli, some of which are not understood. Type II cells detect sweet, bitter, and umami stimuli and Type III cells detect bitter stimuli. The receptor for salt stimuli, which is a focus of the present study, has yet to be fully elucidated (Vandenbeuch, Clapp, & Kinnamon, 2008). It is thought that Type I cells express amiloride-sensitive pathways which are responsible for detection of salty stimuli. These work through the epithelial Na^+ channel or ENaC. These ENaC are required for sodium ion transport across epithelial cells. Sodium salts are detected by direct permeation through apical ion channels, which depolarize the taste cell (Chaudhari & Roper, 2010). A different, amiloride-insensitive pathway is responsible for distinguishing salts aside from NaCl with different cations and anions (Simon, de Araujo, Gutierrez, & Nicolelis, 2006). The amiloride-insensitive pathway is also thought to play a role in salt reception, and the nonselective cation channel TRPV1 has been implicated in this pathway. A study involving TRPV1 knockout mice indicated that while TRPV1 may be responsible for some aversion to NaCl, it is not the primary pathway involved in amiloride-insensitive NaCl taste (Ruiz, Gutknecht, Delay, & Kinnamon, 2006).

The chorda tympani nerve, which innervates fungiform papilla, appears to contain at least two NaCl responsive fibers, N-Type and E-Type fibers. N-type fibers appear to be responsible for the amiloride sensitive transduction pathway as they are responsive to only NaCl and were inhibited by amiloride. E-Type fibers are responsive to various salts such as NaCl, KCl, and HCl and were not inhibited by amiloride. About 44% of taste cells in mice tested in one study were amiloride-sensitive, while 56% of cells were amiloride-insensitive (Yoshida et al., 2009).

Serotonin released from taste buds that acts on 5-HT_3 receptors on nerve fibers has been proposed as the major taste neurotransmitter. However, a study in 2005 by Finger et al. refuted this hypothesis by testing the taste functionality of 5-HT_3 knockout mice. 5-HT_3 knockout mice did not show reduced taste signaling. Instead, ATP has been implicated as the key neurotransmitter involved in transducing taste signals (Finger et al., 2005). Salt taste still proves an anomaly, however, due to the lack of traditional synapses on Type I taste cells. This could be explained by cell-to-cell communication that occurs not only between the taste cells in each taste bud, but also is thought to occur between the different taste buds in the mouth. Since Type II taste cells lack traditional synapses, ATP is also thought to be involved in the cell-to-cell communication between Type II and Type III taste cells. (Huang et al., 2007).

Background on cyclophosphamide

While chemotherapy is often administered as part of a cocktail of drugs, cyclophosphamide (CYP) was one of the first developed chemotherapeutic agents and is still commonly prescribed. Its use includes treatment of leukemia, lymphoma, ovarian, and several other cancers (Mukherjee & Delay, 2011). CYP is a prodrug that acts as a DNA-alkylating agent once inside cells. It forms intra- or interstrand cross linkages in the DNA, which lead to irreparable DNA damage and ultimately apoptosis of the cell (Povirk & Shuker, 1994). Cells

with a high turnover rate are most susceptible to CYP attack, such as hair follicles and cells lining the intestine (Mukherjee & Delay, 2011). In clinical settings, chemotherapy is usually prescribed as a regimen administered on a weekly or monthly basis (DeVita & Chu, 2008). This is done to achieve a sustained concentration of the drug in the body, which can lead to more effective treatment (Bouchard-Fortier et al., 2016).

Similar studies on cyclophosphamide and taste

A past study of the gustatory system following CYP injection showed that umami taste acuity and sensitivity was affected in two phases. The first phase, which occurred 2-4 days post-injection is likely due to cytotoxicity of the CYP on the fungiform taste buds. The second phase, which occurred 9-12 days post-injection is likely due to alteration of the cell replacement system (Mukherjee & Delay, 2011). Taste cells are typically replaced continually, and disturbance to the replacement cycle can lead to long-term taste deficiencies. This study shows that the complaints of altered taste by patients following chemotherapy are not entirely psychological, despite chemotherapy being known to cause long-term cognitive deficits (Ahles & Saykin, 2002). Mukherjee and Delay found that fungiform papillae are most affected after CYP injection, and do not begin recovering until day 12 (Mukherjee & Delay, 2011). Fungiform papillae are located in highest density on the anterior of the tongue and are innervated by the chorda tympani, which is associated with salt taste (Miller & Preslar, 1975). This gave us a further idea of the time-frame in which salt taste is likely affected post CYP administration.

Patients who receive head and neck radiation, another common cancer treatment, also report altered taste. This is not surprising given that both chemotherapy and radiation affect rapidly dividing cells in the body, including taste cells. One proposed cause of this taste dysfunction is that irradiation can damage nerve fibers that innervate taste buds. A second cause

may be loss of salivary glands in the mouth, which could lead to lessened taste acuity. A study by Nguyen, Reyland, and Barlow in 2012 refuted both of these claims, stating that nerve fibers did not appear to be affected by radiation and that xerostomia is not likely to cause such severe taste alteration (Nguyen, Reyland, & Barlow, 2012). Consistent with the Mukherjee and Delay study, Nguyen et al. proposed that radiation affects progenitor cells responsible for supplying new cells to taste buds. Nguyen et al. also noted that cell loss is not immediately apparent due to the existing population of functional taste cells that are not affected by the radiation. However, after the aging population of functional taste cells are lost, they are not immediately replaced due to the damage on the progenitor cells caused by the radiation (Nguyen et al., 2012). This same mechanism likely also applies to chemotherapy due to the similar side effects of each treatment.

Review of investigational methods used

Brief access testing (BAT) is a paradigm used in this study which allows high-throughput testing of mice with altered gustatory function. This technique involves exposing mice to various concentrations of taste stimuli for brief periods of time and recording the numbers of times the mice lick each solution to study the preference for each stimuli (Glendinning, Gresack, & Spector, 2002). Conditioned taste aversion (CTA), a classical conditioning technique, is also used in this study. It involves pairing a conditioned stimulus (CS) with an unconditioned stimulus (US). The CS is typically a taste, while the US is usually lithium chloride (LiCl), which induces an upset stomach in mice. This causes the mice to avoid the CS in the future due to the expectation that CS and US are paired (Lin, Arthurs, & Reilly, 2014).

Study goals

The goal of the present study is to further understand the effects of CYP treatment on the perception of salty taste in mice. The first half of the study used a CTA model to pair NaCl taste with a LiCl injection to induce an aversion to NaCl. After a CYP injection, the aversion was evaluated for changes. The second half of the study used a simple BAT model to compare the different effects of a single CYP dose versus multiple CYP doses over several days to better replicate a clinical CYP administration. Generally, our hypothesis was that CYP treatment would affect Type I taste cells, leading to changes in salt preference. We further hypothesized that CYP treatment would reduce salt aversion. Lastly, we hypothesized that multiple doses over time would affect multiple salt taste cell populations leading to more significant changes in salt preference.

Chapter II: CTA Study

Introduction

Conditioned taste aversion (CTA) methodology involves pairing a novel taste, known as the conditioned stimulus (CS), with a treatment, known as the unconditioned stimulus (US), which induces illness. Animals will then avoid the CS due to the assumption that the CS and the US are paired (Lin et al., 2014). CTA methodology can be useful in a range of studies, including evaluating the change in an aversion after a treatment. While conducting CTA work, it is important to consider that a period of extinction occurs when an animal is exposed to only the CS and not the US, which causes the animal to lose the aversion (Hadamitzky, Bösche, Engler, Schedlowski, & Engler, 2015).

By inducing a CTA to NaCl in mice, then treating them with CYP, changes in the salt aversion afterwards can be used to infer the underlying cellular effects of CYP. The two different populations of Type I taste cells, one group with a lifespan of about 8 days and another with a life span of about 24 days, give us a time frame needed to fully evaluate how Type I taste cell populations change over time following CYP treatment (Perea-Martinez, Nagai, & Chaudhari, 2013). Mukherjee and Delay found that CYP treatment lowers umami taste acuity and sensitivity (Mukherjee & Delay, 2011). This led us to hypothesize that CYP treatment will reduce salt aversion.

Methods

Two pilot studies were conducted to evaluate testing conditions for mice including ideal NaCl concentrations and length of testing sessions. Based on these studies, NaCl became naturally aversive between 175-300 mM, giving us a NaCl concentration range. The following methods were based on those two studies and other relevant studies in the field.

Subjects

Thirty-two male C57BL/6J mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were used in this study. Mice were housed in groups of four with their littermates, unless a mouse became sick, in which case it was moved to a separate solitary cage for treatment and observation. Mice were monitored by The University of Vermont veterinary technicians for the entirety of the study. The room was kept at 25° C and 60% humidity. Food pellets were provided ad libitum. All mice were ordered at six to eight weeks of age and allowed to acclimate to the room for at least one week. Mice were kept on a water deprivation schedule of 23 hours per day throughout the study which started one week before the beginning of the study. Mice were also handled during this initial water deprivation period to socialize them before experimentation. Any mouse that became too ill at any point in the experiment was immediately removed from the study and received veterinary attention. All mice were euthanized at the end of the study. All procedures were approved by The University of Vermont IACUC, protocol 10-038.

Apparatus

Licks were monitored by computer-operated Davis Rig lickometers (DiLog Instruments, Tallahassee, FL, USA). Each Davis Rig consisted of a chamber (30cm x 15 cm x 23 cm) and a sliding block which could hold up to 16 glass tubes with lick spouts containing taste stimuli.

Tubes were filled with fresh NaCl solutions of varying concentrations and washed daily. The block containing the tubes ran based on a program on the computer. Each of the four Davis Rigs was separated from the other by a wooden box and a curtain, and pink noise was produced from a speaker in each box. Each tube was approximately 2mm behind a shutter. After a six second inter presentation interval period, the shutter opened and the mouse was given a chance to lick the solution for 7.5 seconds. Each time the mouse made contact with the metal lick spout, the computer recorded a lick. If the mouse did not lick a given solution, the shutter remained open until the mouse began to lick or the 20-minute time limit ran out.

Davis Rig Habituation (5-7 days)

Mice were habituated to the Davis Rigs with water-only trials. Sessions started at 12pm daily and lasted 20 minutes. Water tubes were presented in the manner described above and would end after 20 minutes. Three water tubes were presented in a randomized order to allow the mice to habituate to the movement of the block. Habituation continued until mice were consistently licking, which on average took five days.

Conditioning (5-7 days)

Half of the mice were conditioned to avoid NaCl. Mice were presented with 25, 50, 100, 175, and 300 mM NaCl solutions along with two water tubes in a randomized order. Two random water trials from each mouse on each day were averaged giving us a "0 mM NaCl" water rinse value. Latin-Square procedures were used to generate random tube sequences. Each trial began with four presentations of water, which acted as a rinse for the mice. Immediately following the twenty minute sessions, the conditioned mice were injected intraperitoneally with 225 mM LiCl (1mL/kg) dissolved in bacteriostatic water to induce an upset stomach, leading to

an aversion to NaCl. The remaining mice acted as a control and were injected with saline (1ml/kg) which does not induce an aversion. This process was repeated daily at 12pm until LiCl mice showed a clear aversion to NaCl. An aversion was identified as a NaCl lick rate for each mouse of <40% when compared to the water licks for that mouse.

Cyclophosphamide Injection

Mice were taken off water deprivation for 24 hours to rehydrate them in preparation for CYP injection. At 12pm the following day, half of the LiCl mice and half of the control mice were injected intraperitoneally with 100 mg/kg CYP, and the remaining mice received a saline injection. 24 hours later, the mouse cages were changed to prevent them from becoming ill due to the toxic CYP byproducts secreted in urine after injection. At this time, the mice were also returned to a 23-hour water deprivation schedule so that testing could begin the following day.

NaCl Testing (20-25 Days)

Next, mice were presented with the same 25, 50, 100, 175, and 300 mM NaCl solutions as described above in the conditioning step. About an hour after each session, the mice were given water bottles for approximately five minutes to rehydrate after the high salt intake during the session.

Statistical Methods

Lick rates were normalized by dividing the mean lick count for each concentration of NaCl by the mean lick count for the water trials. This step was taken to ensure that variable motivational states between mice was accounted for in our analysis. Some graphs show

normalized lick rates on a 100-point scale, which is the previously described normalized lick rate multiplied by 100. Any mouse who did not get through at least one presentation of each NaCl concentration on any given day would have its data excluded for that day. The sample size of each group was eight from day 2-21. From day 22-25, the sample size for all groups was four. This is due to a decision to run the second group of mice longer to ensure all CYP effects were observed in the study time frame.

Ensuring proper condition before testing

CTA was considered successful when LiCl injected mice show significantly fewer licks than control mice. A 2 (US) x 5 (Five concentrations of NaCl) mixed factorial analysis was used to analyzed the lick rates for each group of mice. These were computed using IBM SPSS Statistics 23 software.

Changes in salt preference following injection

Our analysis used a mixed factorial design, with two treatment condition states (Saline and LiCl) by two drug states (CYP and Control) as subject variables. Within the subject variables, there were five concentrations (25, 50, 100, 175, and 300 mM NaCl) and 25 days. Because of the large number of days, data were partitioned for specificity and ease of analysis. Averages of lick rates for each concentration on each day for each mouse were used in the following analyses.

Results

Pre-CYP group differences

Pre-CYP injection group differences between both groups of LiCl mice were not significant for any concentration of NaCl. Pre-CYP injection group differences between both groups of non-conditioned mice were not significant for any concentration of NaCl ($p>0.05$).

Did the CYP have an effect on NaCl licks?

A condition state (LiCl or Saline) by drug state (CYP or Saline) by day analysis revealed significantly higher licks for mice who received CYP compared to control mice at 50, 100, 175, and 300 mM NaCl as seen in Table 1 ($p <0.005$). The effect of the drug for 25 mM NaCl was not significant. Graphs of each concentration over days are shown in Figures 1-6. The drug state by day interaction was not statistically significant for any concentration.

Drug State	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	25	0.800	0.027	(1,149) = 2.80	0.371
CYP		0.834	0.026		
Control	50	0.684	0.025	(1,151) = 0.81	**0.017**
CYP		0.768	0.024		
Control	100	0.614	0.025	(1,143) = 7.68	**0.006**
CYP		0.708	0.024		
Control	175	0.532	0.025	(1,133) = 27.15	**<0.001**
CYP		0.712	0.024		
Control	300	0.499	0.031	(1,108) = 18.80	**<0.001**
CYP		0.686	0.030		

Table 1: Condition state by drug state by day analysis comparing the control group and the CYP group. CYP mice show significantly higher lick rates for 50, 100, 175, and 300 mM NaCl.

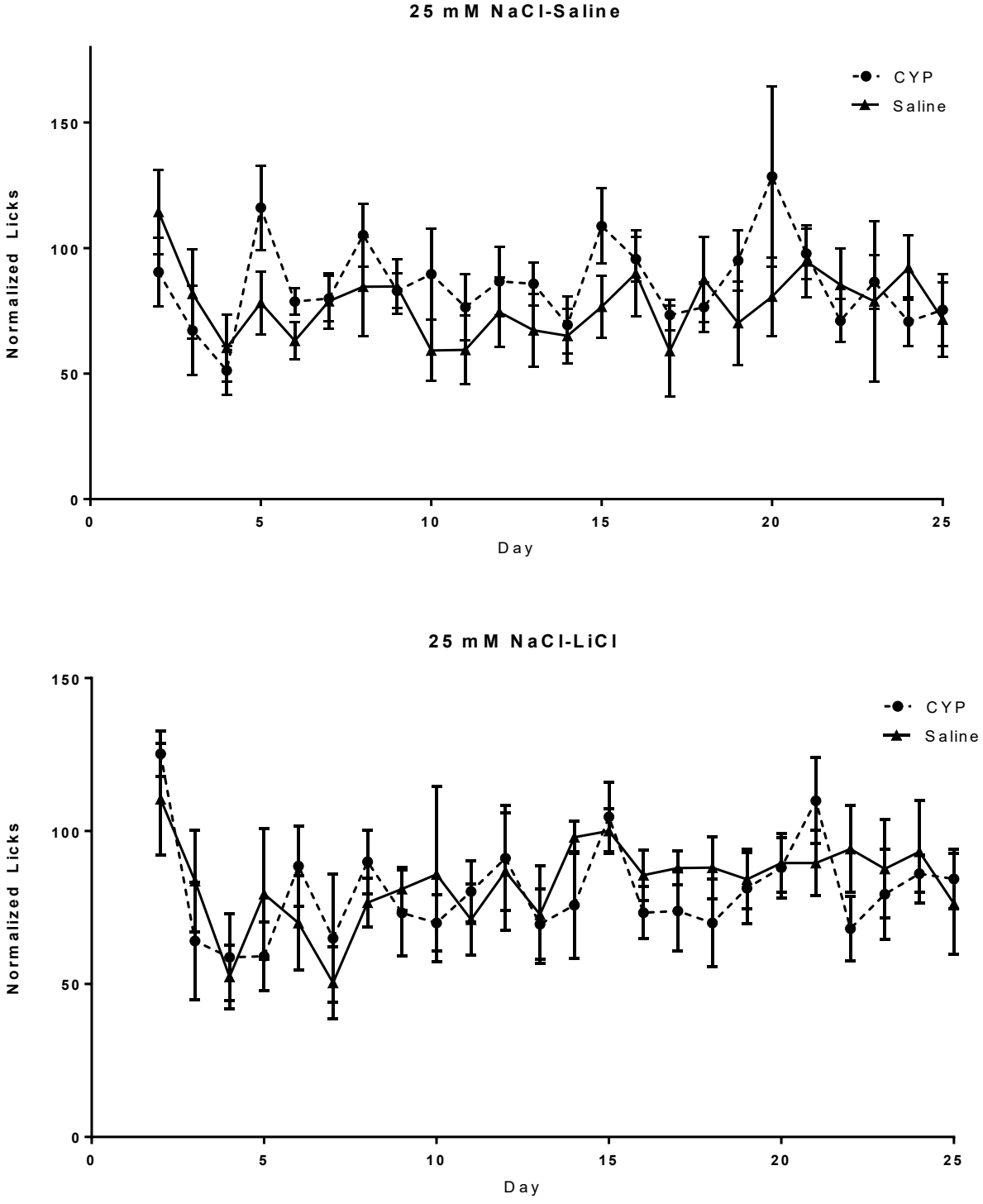

Figure 1: Normalized lick rates over days for 25 mM NaCl. The upper graph shows mice that were not conditioned to avoid NaCl, while the lower graphs shows mice that were conditioned to avoid NaCl. No significant drug effects are seen in the Saline/Saline vs. Saline/CYP conditions (p>0.05), seen in the upper graph, or the LiCl/CYP vs. LiCl/Saline conditions (p>0.05), seen in the lower graph.

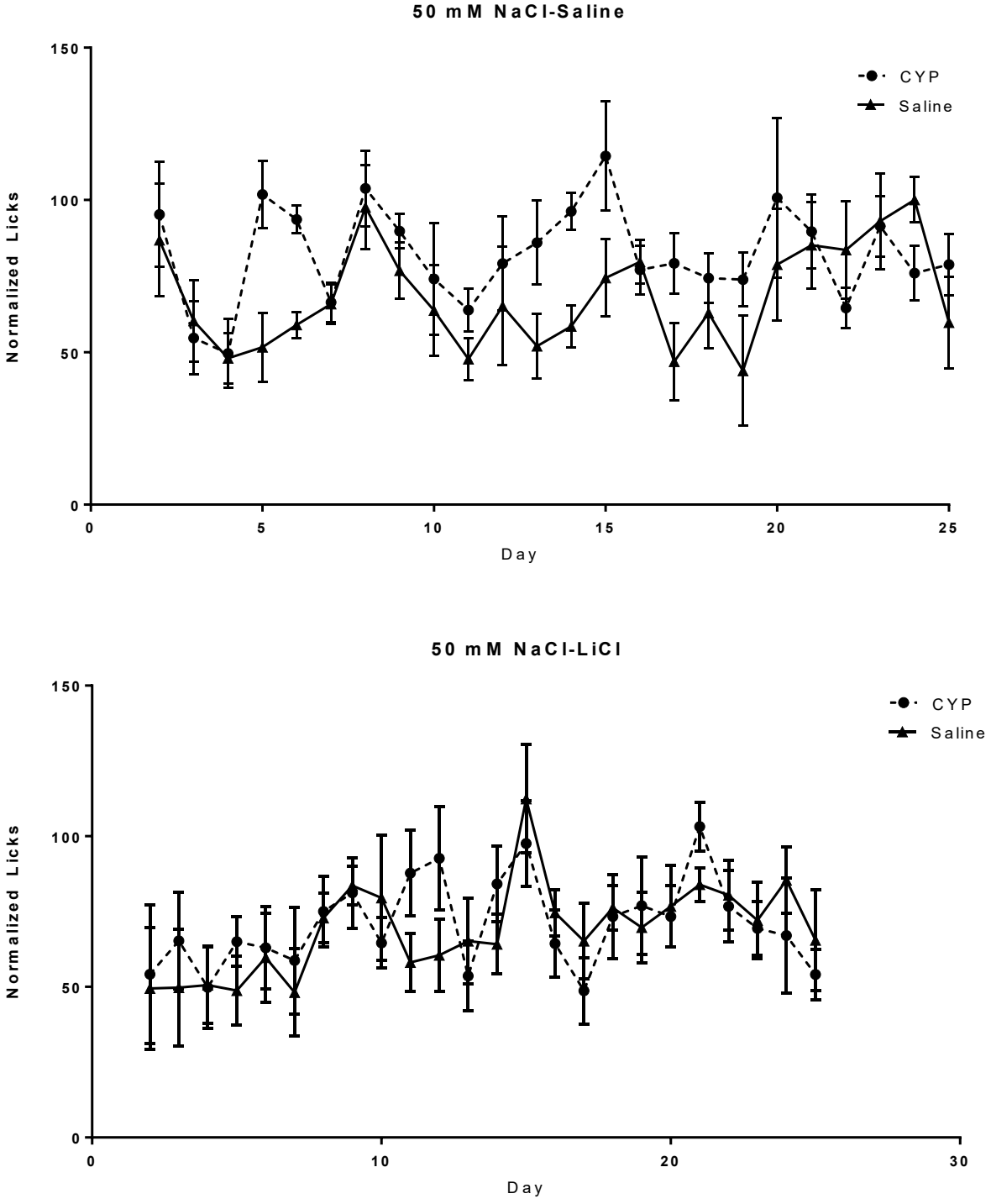

Figure 2: Normalized lick rates over days for 50 mM NaCl. The upper graph shows mice that were not conditioned to avoid NaCl, while the lower graphs shows mice that were conditioned to avoid NaCl. CYP mice licked significantly more than control mice Saline/Saline vs. Saline/CYP conditions ($p = 0.002$), seen in the upper graph. There were no significant drug effects between the LiCl/CYP vs. LiCl/Saline conditions ($p > 0.05$), seen in the lower graph.

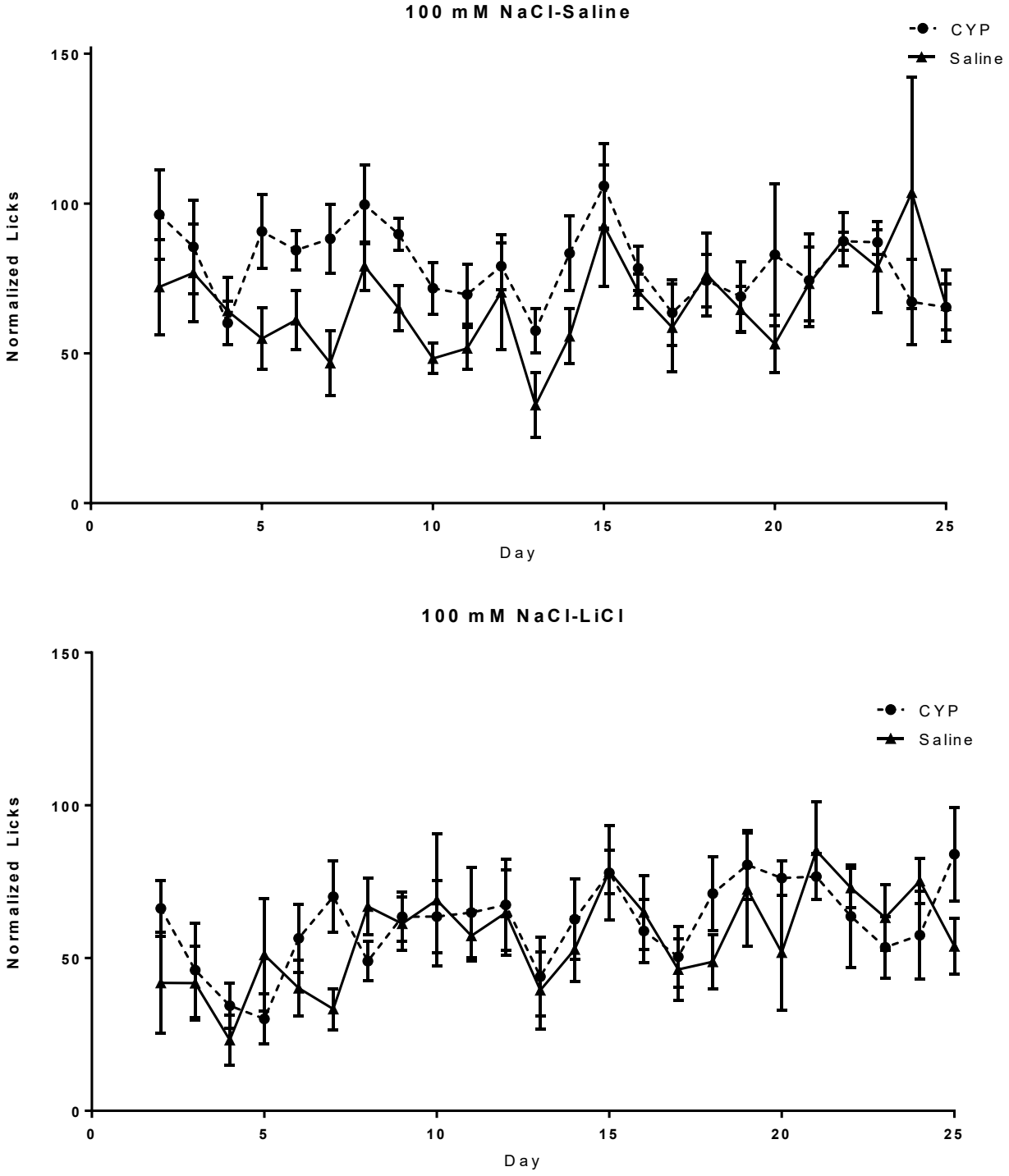

Figure 3: Normalized lick rates over days for 100 mM NaCl. The upper graph shows mice that were not conditioned to avoid NaCl, while the lower graphs shows mice that were conditioned to avoid NaCl. CYP mice licked significantly more than control mice Saline/Saline vs. Saline/CYP conditions ($p = 0.003$), seen in the upper graph. There were no significant drug effects between the LiCl/CYP vs. LiCl/Saline conditions ($p>0.05$), seen in the lower graph.

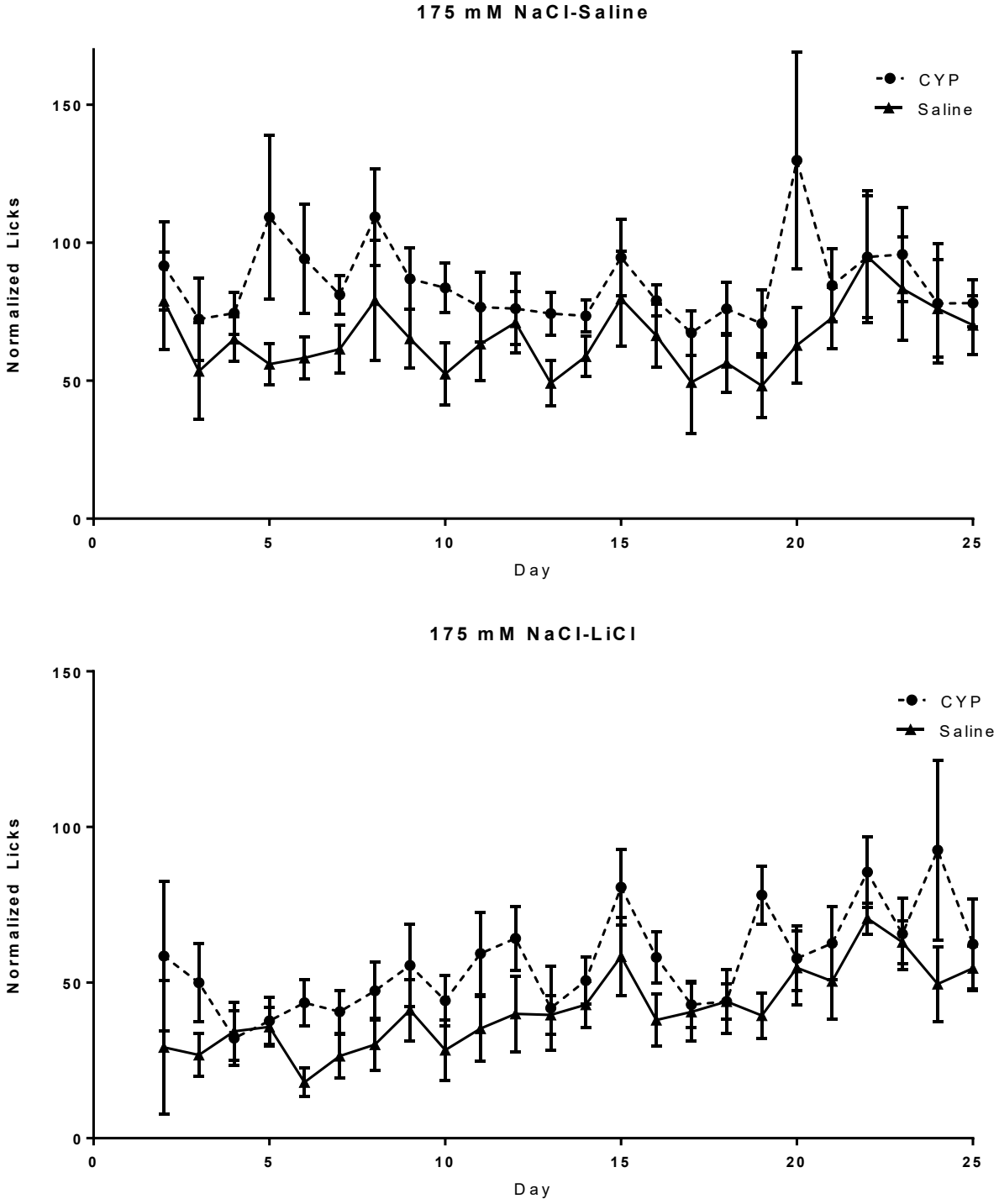

Figure 4: Normalized lick rates over days for 175 mM NaCl. The upper graph shows mice that were not conditioned to avoid NaCl, while the lower graphs shows mice who that were conditioned to avoid NaCl. CYP mice licked significantly more in the Saline/Saline vs. Saline/CYP conditions, ($p = 0.001$), seen in the upper graph, and the LiCl/CYP vs. LiCl/Saline conditions ($p < 0.001$), seen in the lower graph.

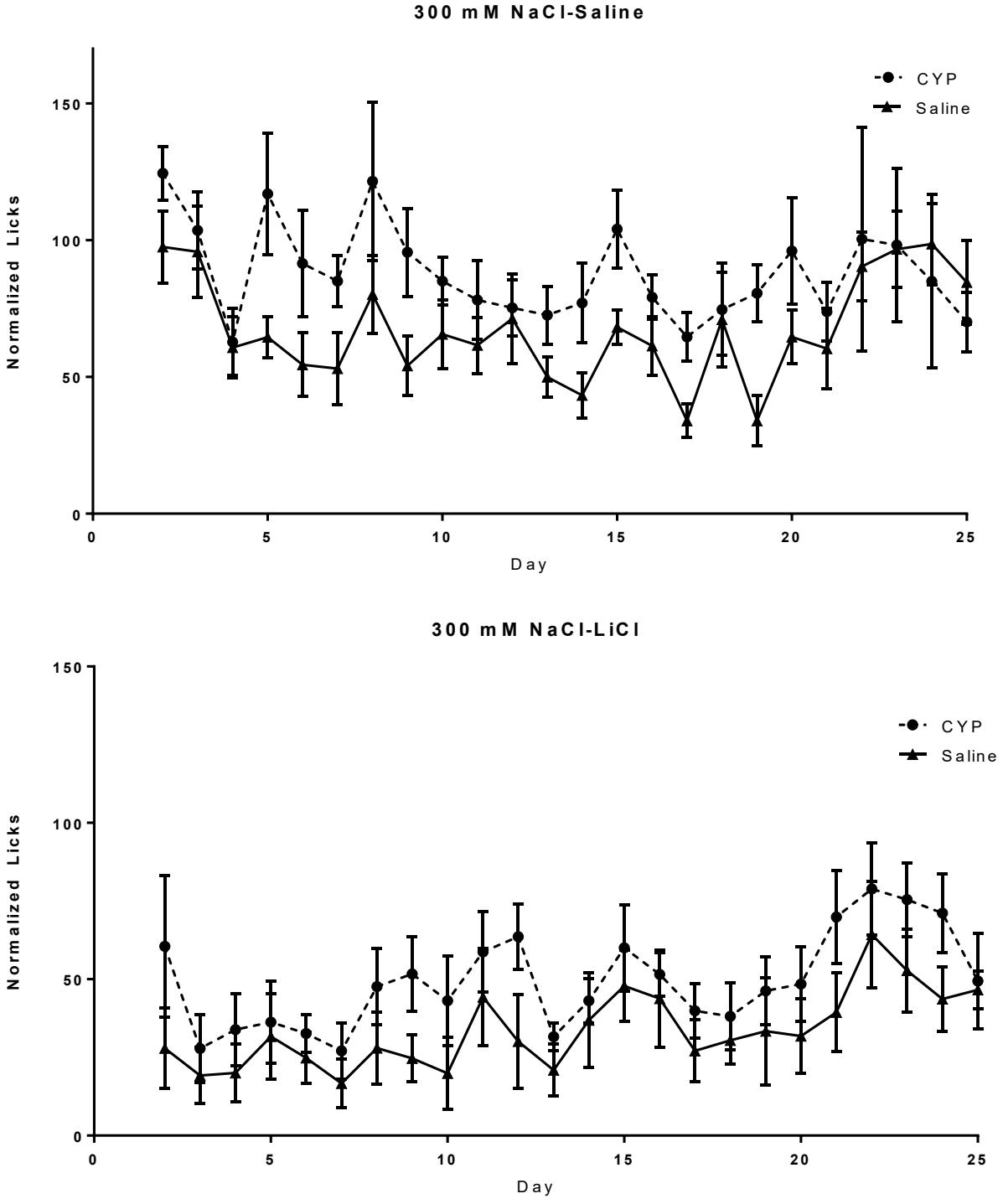

Figure 5: Normalized lick rates over days for 175 mM NaCl. The upper graph shows mice that were not conditioned to avoid NaCl, while the lower graphs shows mice that were conditioned to avoid NaCl. CYP mice licked significantly more in the Saline/Saline vs. Saline/CYP conditions, (p = 0.001), seen in the upper graph, and the LiCl/CYP vs. LiCl/Saline conditions (p =0.011), seen in the lower graph.

Non-conditioned vs. Conditioned Mice

To confirm proper conditioning, the NaCl lick rates of LiCl mice were compared to the NaCl lick rates of non-conditioned mice. LiCl mice showed significantly fewer licks for 100 mM NaCl, $F(1, 143) = 18.19$, $p < 0.001$, 175 mM NaCl, $F(1, 133) = 60.54$, $p < 0.001$, and 300 mM, $F(1, 108) = 71.60$, $p < 0.001$, when compared to non-conditioned mice.

A drug state by day analysis revealed that non-conditioned mice who received CYP had significantly higher lick rates across more NaCl concentrations than LiCl mice when compared to control mice. Non-conditioned, CYP mice showed significantly higher lick rates for 25, 50, 100, 175, and 300 mM NaCl compared to control mice, seen in Table 2. LiCl, CYP mice showed significantly higher licks for 175 and 300 mM NaCl compared to control mice, seen in Table 3.

Condition State	Drug State	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Saline	Control	25	0.770	0.041	(1,67) = 2.74	0.103
	CYP		0.862	0.038		
Saline	Control	50	0.682	0.033	(1,80) = 10.20	**0.002**
	CYP		0.824	0.030		
Saline	Control	100	0.666	0.032	(1,75) = 9.36	**0.003**
	CYP		0.801	0.030		
Saline	Control	175	0.653	0.045	(1,59) = 11.56	**0.001**
	CYP		0.860	0.041		
Saline	Control	300	0.669	0.047	(1,58) = 11.38	**0.001**
	CYP		0.883	0.043		

Table 2: Drug state by day analysis comparing the control group and the CYP group in the non-conditioned state. CYP mice showed significantly higher lick rates for 50, 100, 175 and 300 mM NaCl.

Condition State	Drug State	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
LiCl	Control	25	0.830	0.036	(1,82) = 0.230	0.633
	CYP		0.806	0.036		
LiCl	Control	50	0.688	0.037	(1,72) = 0.170	0.681
	CYP		0.710	0.037		
LiCl	Control	100	0.563	0.036	(1,69) = 0.979	0.326
	CYP		0.614	0.036		
LiCl	Control	175	0.411	0.026	(1,18) = 17.50	**<0.001**
	CYP		0.565	0.026		
LiCl	Control	300	0.328	0.042	(1,48) = 7.09	**0.011**
	CYP		0.487	0.042		

Table 3: Drug state by day analysis comparing the control group and the CYP group in the LiCl conditioned state. CYP mice showed significantly higher lick rates for 175 and 300 mM NaCl.

Specific days of interest

A condition state by drug state by concentration analysis done on each individual day revealed certain days when drug effects were significant to test if there was a CYP effect pattern or cycle. CYP mice shower significantly higher lick rates compared to control mice on days 5, 6, 7, 9, 11, 13, seen in Table 4 and Figure 7. No significant drug effects were seen on days 14-18, then CYP mice again showed significantly higher lick rates on day 19 seen in Figure 8. Drug effects then remained insignificant for the remainder of the study (Day 20-25).

Drug State	Day	Mean normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	5	0.590	0.064	(1,37) = 5.81	**0.021**
CYP		0.879	0.062		
Control	6	0.583	0.049	(1,33) = 8.20	**0.007**
CYP		0.779	0.048		
Control	7	0.539	0.057	(1,33) = 5.37	**0.027**
CYP		0.719	0.053		
Control	9	0.698	0.035	(1,44) = 5.43	**0.024**
CYP		0.815	0.036		
Control	11	0.597	0.042	(1,43) = 5.85	**0.020**
CYP		0.740	0.042		
Control	13	0.538	0.046	(1,114) = 10.30	**0.021**
CYP		0.691	0.044		
Control	19	0.612	0.049	(1,42) = 5.90	**0.019**
CYP		0.778	0.048		

Table 4: Condition state by drug state by concentration analysis comparing control mice and CYP mice. CYP mice showed significantly higher lick rates on days 5, 6, 7, 9, 11, 13, and 19. No significant drug effect was seen on the other days.

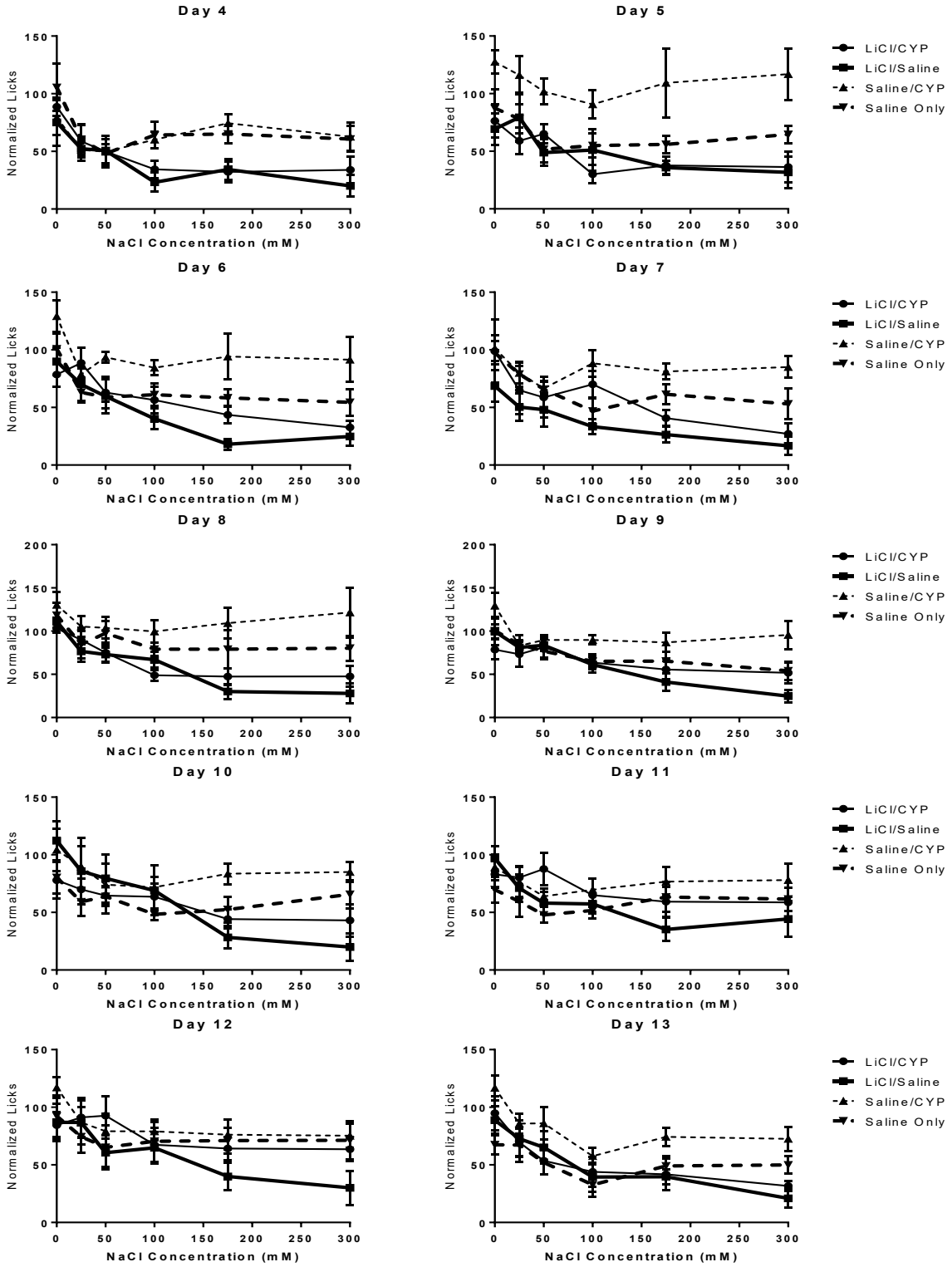

Figure 6: Normalized lick rates for days 4-13. Non-conditioned mice have dotted lines, while LiCl, conditioned mice have solid lines. Drug state control mice (saline) have bolded lines. CYP mice had significantly higher lick rates on days 5, 6, 7, 9, 11, and 13 ($p<0.05$). Days 4, 8, 10, and 12 were included for comparison.

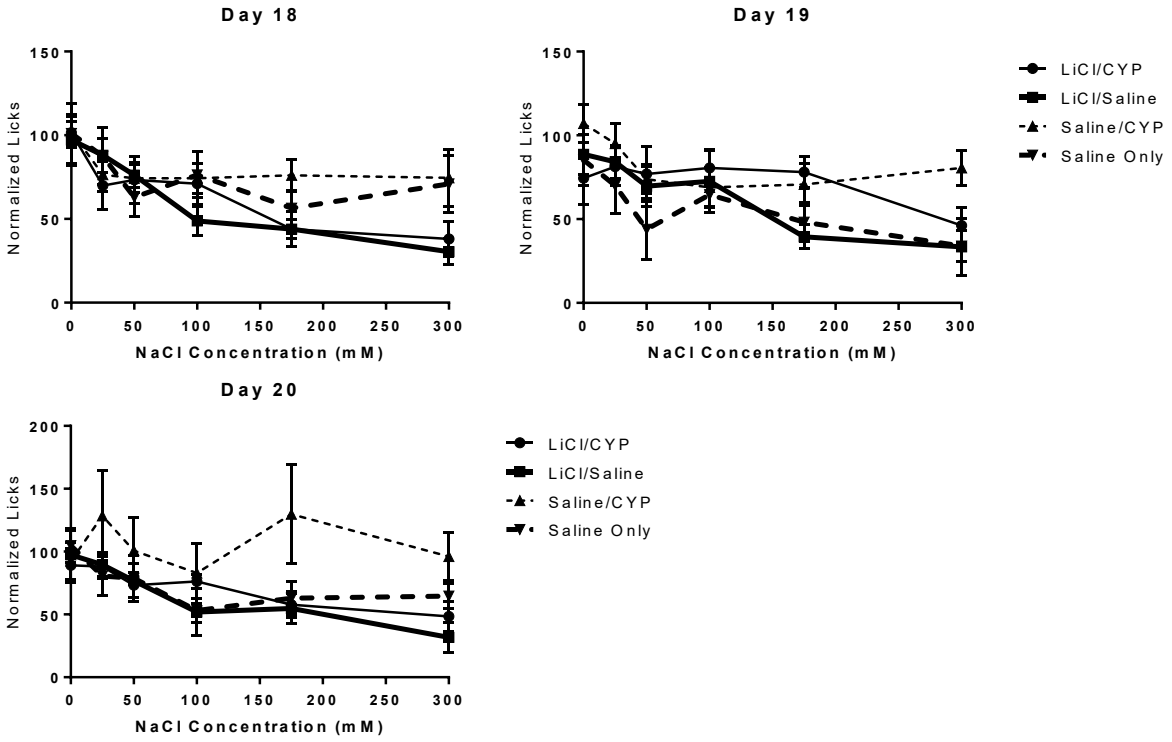

Figure 7: Normalized lick rates for days 18-20. Non-conditioned mice have dotted lines, while LiCl, conditioned mice have solid lines. Drug state control mice (saline) have bolded lines. CYP mice had significantly higher lick rates on day 19 (p =0.019). Days 18 and 20 were included for comparison.

A drug state by day analysis of conditioned LiCl mice revealed that LiCl mice had no significant drug effects on any day. In contrast, non-conditioned, CYP injected mice showed significantly higher lick rates compared to control mice on days 5, 6, 7, 9, 13, and 14, seen in Table 5 and in Figure 6. Significant drug effects were not seen again until day 19, when non-conditioned CYP mice had significantly higher lick rates compared to controls, seen in Figure 7.

Condition State	Drug State	Day	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Saline	Control	5	0.689	0.109	(1,18) = 9.23	**0.007**
	CYP		1.143	0.102		
Saline	Control	6	0.679	0.070	(1,15) = 0.910	**0.009**
	CYP		0.976	0.070		
Saline	Control	7	0.682	0.059	(1,20) = 4.52	**0.046**
	CYP		0.848	0.051		
Saline	Control	9	0.750	0.074	(1,15) = 5.60	**0.032**
	CYP		0.991	0.070		
Saline	Control	13	0.541	0.065	(1,21) = 11.45	**0.003**
	CYP		0.844	0.061		
Saline	Control	14	0.660	0.057	(1,21) = 6.56	**0.018**
	CYP		0.861	0.054		
Saline	Control	19	0.581	0.079	(1,20) = 6.13	**0.022**
	CYP		0.850	0.074		

Table 5: Drug state by day analysis comparing non-conditioned mice and control mice. CYP mice showed significantly higher lick rates on days 5, 6, 7, 9, 13, 14, and 19.

In summary, CYP mice showed significantly higher lick rates compared to control mice at 50, 100, 175, and 300 mM NaCl. Non-conditioned mice showed significantly higher lick rates compared to control mice at more NaCl concentrations than LiCl mice. CYP mice showed significantly higher lick rates compared to control mice on days 5, 6, 7, 8, 11, and 19.

Discussion

In this study, we attempted to use CTA methodology to evaluate the effect of CYP on salt taste in mice. We hypothesized that CYP treatment would affect Type I taste cells, leading to changes in salt preference. We also hypothesized that CYP treatment would reduce salt aversion.

CYP injected LiCl mice, who were conditioned to avoid NaCl, generally showed higher lick rates for both 175 mM and 300 mM compared to the control group throughout the study. Higher licks rates indicate reduced aversion, which supports our reduced aversion hypothesis.

However, mice that were not conditioned to avoid NaCl and that were treated with CYP showed significantly higher NaCl lick rates at more concentrations than the LiCl mice when compared to the control group. These non-conditioned CYP mice showed significantly higher lick rates at 50, 100, 175, and 30 mM NaCl. This discrepancy could be explained by the presence of TRPV1 which has been proposed to be involved with aversion to NaCl (Ruiz et al., 2006). CYP may be affecting Type I taste cells, but if a secondary salt taste cell type is unaffected by CYP, an aversion would still remain after treatment. TRPV1 cells, which may be involved with NaCl aversion, may not be sensitive to CYP treatment. Even though TRPV1 are not the predominate secondary salt reception cell, the second population of salt receptor cells could have a vastly different life span than Type I taste cells (Ruiz et al., 2006). A second possibility for why LiCl mice showed drug effects at fewer concentrations could involve this difference in life span. We know that CYP mainly affects dividing cells (Mukherjee & Delay, 2011). By the time the amiloride-insensitive cells need to be replaced by CYP damaged progenitor cells, the aversion to NaCl could be extinct. This secondary disturbance could explain the day 19 significant drug effect seen in LiCl mice in Figure 5.

Still, non-conditioned mice who received CYP injections showed significantly higher lick rates for most of the concentrations of NaCl. Both preliminary pilot studies and our saline only control groups indicate that NaCl becomes naturally aversive between 175 and 300 mM. Our data suggest that drug effects start around day 5 post-CYP treatment and continue to around day 13, then occur again around day 19. This two-phase disturbance could coincide with the different life spans of Type I taste cells, three-fourths of which have a half-life of eight days, while the other live for 24 days (Perea-Martinez et al., 2013).

Chapter III: Multiple vs. One Dose of CYP

Introduction

Results from our CTA study indicate that LiCl, CYP mice did not show as many concentrations of significance compared to non-conditioned mice. For this reason, the CTA aspect of our next study was removed and replaced with a simple BAT paradigm, where mice are exposed to taste solutions for a short period of time without LiCl injections. Due to the interest in a secondary salt receptor cell population, we developed a methodology aimed at disturbing multiple salt receptor cell types (Yoshida et al., 2009).

Preliminary molecular studies in our lab indicate that multiple CYP injections over days could disturb multiple salt receptor cell populations. This would more closely replicate chemotherapy regimens that are used in a clinical setting (DeVita & Chu, 2008). Thus, a study involving both multiple and single injection mice may help to elucidate not only the overall effect that CYP has on salt taste, but also uncover the CYP sensitivity of other salt sensitive cell types as well. We hypothesized that multiple CYP doses over time would affect multiple salt receptor cell types leading to more significant changes in salt preference.

Methods

Subjects

Thirty-one male C57BL/6J mice obtained from Jackson Laboratory (Bar Harbor, ME, USA) were used in this study. Mice were housed in groups of four with their littermates, unless a mouse became sick in which case it was moved to a separate solitary cage for treatment and observation. Mice were monitored by The University of Vermont veterinary technicians for the entirety of the study. The room was kept at 25° C and 60% humidity. Food pellets were provided

ad libitum. All mice were ordered at six to eight weeks of age and allowed to acclimate to the room for at least one week. Mice were kept on a water deprivation of 23 hours throughout the study which started one week before the beginning of the study. Mice were also handled during this initial water deprivation period to socialize them before experimentation. Any mouse that became too ill at any point in the experiment was immediately removed from the study and received veterinary attention. All mice were euthanized at the end of the study. All procedures were approved by The University of Vermont IACUC, protocol 10-038.

Apparatus

Licks were monitored by computer-operated Davis Rig lickometers (DiLog Instruments, Tallahassee, FL, USA). Each Davis Rig consisted of a chamber (30cm x 15 cm x 23 cm) and a sliding block which could hold up to 16 glass tubes with lick spouts containing taste stimuli. Tubes were filled with fresh NaCl solutions of varying concentrations and washed daily. The block containing the tubes ran based on a program on the computer. Each Davis Rig was separated from the other by a wooden box and a curtain, and pink noise was produced from a speaker in each box. Each tube was approximately 2mm behind a shutter. After a 6 second inter presentation interval period, the shutter opened and the mouse was given a chance to lick the solution for 7.5 seconds. Each time the mouse made contact with the metal lick spout, the computer recorded a lick. If the mouse did not lick a given solution, the shutter remained open until the mouse began to lick or the 20-minute time limit ran out.

Davis Rig Habituation (5-7 days)

Mice were habituated to the Davis Rigs with water-only trials. Sessions started at 12pm daily and lasted 20 minutes. Water tubes were presented in the manner described above and

would end after 20 minutes. Three water tubes were presented in a randomized order to allow the mice to habituate to the movement of the block. Habituation continued until mice were consistently licking, which on average took five days.

Initial NaCl Presentation (~5 Days)

Mice were presented with 50, 100, 175, and 300 mM NaCl solutions along with two water tubes in a randomized order. Sucrose (100 mM), which is typically licked at a higher rate than water by mice, was also included in the sequence to test for neophobia (the fear of new things). Latin-Square procedures were used to generate random tube sequences. Each trial began with four presentations of water which acted as a rinse for the mice. This process was repeated daily at 12pm until mice showed a consistent NaCl licking pattern. This typically took about 5 days.

Cyclophosphamide Injection

Mice were taken off water deprivation for 24 hours to rehydrate them in preparation for CYP injection. One third of the mice were assigned multiple injections, another third were assigned a single injection, and the remaining mice acted as control mice who received saline injections. At 12pm the following day, the multiple injection mice received a 20 mg/kg dose of CYP intraperitoneally and the remaining mice received the same volume of saline. This continued for five days, and on the fifth day, the multiple injection mice received a 20 mg/kg dose as before, the single injection mice received one 100 mg/kg intraperitoneal CYP injection, and the control mice received a saline injection of the same volume as the other mice. 24 hours later, the mouse cages were changed to prevent them from becoming ill due to the toxic CYP

byproducts secreted in urine after injection. At this time, the mice were also returned to the 23-hour water deprivation schedule so that testing could begin the following day.

NaCl Testing (31 Days)

Next, mice were presented with the same 50, 100, 175, and 300 mM NaCl and 100 mM sucrose solutions as described above in the initial NaCl presentation step. An hour after each session, the mice were given water bottles for approximately five minutes to rehydrate due to the high salt intake during the session.

Statistical Methods

Lick rates were normalized by dividing the mean lick count for each concentration of NaCl by the mean lick count for the water trials. This step was taken to ensure that variable motivational states between mice was accounted for in our analysis. Some graphs show normalized lick rates on a 100-point scale, which is the previously described normalized lick rate multiplied by 100. Any mouse who did not get through at least one presentation of each NaCl concentration on any given day would have its data excluded for that day. The number of mice in each group is 10 from days 2-15. The remaining mice are still being run, so the number of mice from day 16-31 is only five.

Changes in salt preference following injection

Our analysis used a mixed factorial design, with two injection states (Single or multiple) by two drug states (CYP and Control) as subject variables. Within the subject variables, there were four concentrations (50, 100, 175, and 300 mM NaCl) and 31 days. Because of the large

number of days, data were partitioned for specificity and ease of analysis. Averages of lick rates for each concentration on each day for each mouse were used in the following analyses. These analyses were done using IBM SPSS Statistics 23 software.

Results

Pre-CYP group differences

Before CYP injection, group differences between the three conditions were not significant for any concentration of NaCl ($p>0.05$).

Neophobia (Fear of new things)

All mice had mean lick rates for 100 mM sucrose that exceeded mean water lick rates. The average for all mice was a normalized lick rate of 142% compared to water.

Drug effects

In a drug condition (CYP or Control) by day analysis, CYP injected mice had higher normalized lick rates compared to control mice for 175 mM NaCl, seen in Table 6. The drug state by day interaction was also significant for 175 mM NaCl, $F(29, 321) = 1.51$, $p = 0.048$, but not for any other concentration.

Drug State	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	50	0.680	0.027	(1,169) = 0.21	0.649
CYP		0.695	0.016		
Control	100	0.649	0.026	(1,165) = 0.174	0.677
CYP		0.661	0.016		
Control	175	0.577	0.025	(1,162) = 6.75	**0.010**
CYP		0.651	0.015		
Control	300	0.735	0.031	(1,140) = 0.13	0.724
CYP		0.722	0.018		

Table 6: Drug state by day analysis comparing the control mice and CYP injected mice. CYP mice showed significantly higher lick rates for 175 mM NaCl.

Multiple Doses vs. Single Dose

A dosage by day analysis revealed that there was no significant difference in lick rates for mice who received a single CYP injection compared to mice who received multiple CYP injections at any concentration, seen in Table 7.

Dosage	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Single	50	0.697	0.022	(1,123) = 0.02	0.897
Multiple		0.693	0.020		
Single	100	0.661	0.024	(1,107) = 0.00	0.952
Multiple		0.663	0.021		
Single	175	0.648	0.022	(1,111) = 0.08	0.774
Multiple		0.656	0.020		
Single	300	0.695	0.026	(1,96) = 2.08	0.153
Multiple		0.746	0.024		

Table 7: Dosage by day analysis comparing the single CYP injection group and the multiple CYP injection group. The group differences were not significant at any concentration.

Single CYP injection mice showed significantly higher lick rates compared to control mice at 175 mM, shown in Table 8 and Figure 8. Multiple CYP injection mice showed significantly higher lick rates compared to control mice at 175 mM, seen in Table 9 and Figure 8.

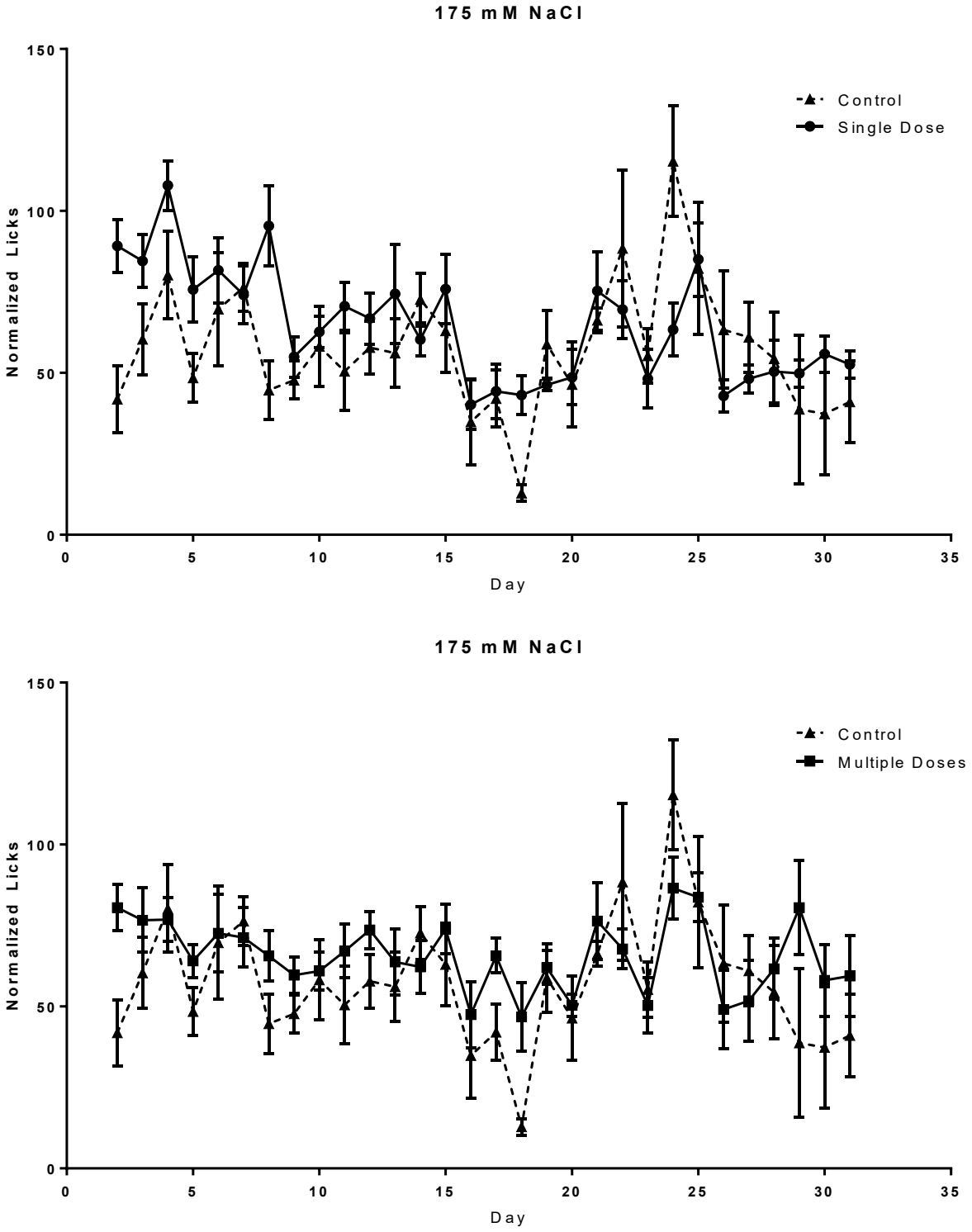

Figure 8: Normalized lick rates over days for 175 mM NaCl. The upper graph shows single CYP dose mice compared to control mice, while the lower graph shows multiple CYP dose mice compared to control mice. Both single injection mice and multiple injection mice showed significantly higher lick rates overall when compared to control mice at 175 mM NaCl ($p<0.05$).

Dosage	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	50	0.680	0.028	(1,98) = 0.19	0.665
Single		0.696	0.025		
Control	100	0.648	0.027	(1,96) = 0.95	0.759
Single		0.660	0.024		
Control	175	0.577	0.024	(1,95) = 4.80	**0.031**
Single		0.647	0.022		
Control	300	0.734	0.029	(1,82) = 1.03	0.313
Single		0.695	0.026		

Table 8: Dosage by day analysis comparing the control group and single CYP injection group. Single injection mice showed significantly higher lick rates for 175 mM NaCl.

Dosage	NaCl Concentration (mM)	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	50	0.681	0.029	(1,105) = 0.11	0.745
Multiple		0.693	0.024		
Control	100	0.649	0.027	(1,108) = 0.00	0.688
Multiple		0.663	0.022		
Control	175	0.577	0.025	(1,111) = 6.10	**0.015**
Multiple		0.656	0.020		
Control	300	0.734	0.032	(1,93) = 0.08	0.783
Multiple		0.746	0.026		

Table 9: Dosage by day analysis comparing the control group and multiple CYP injection group. Multiple injection mice showed significantly higher lick rates for 175 mM NaCl.

Specific Days of Interest

A drug state by concentration analysis performed on each individual day revealed days of interest. Mice injected with CYP showed significantly higher lick rates compared to control mice on days 3, 5, 8, and 18, seen in Table 10. Control mice showed significantly higher lick rates compared to CYP mice on day 22, also seen in Table 10.

Drug State	Day	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	3	0.866	0.056	(1,49) = 7.51	**0.009**
CYP		1.046	0.034		
Control	5	0.769	0.048	(1,48) = 4.98	**0.030**
CYP		0.895	0.030		
Control	8	0.812	0.065	(1,60) = 7.61	**0.008**
CYP		1.026	0.042		
Control	18	0.479	0.060	(1,22) = 11.17	**0.003**
CYP		0.715	0.036		
Control	22	1.136	0.063	(1,28) = 8.01	**0.009**
CYP		0.926	0.038		

Table 10: Drug state by concentration analysis comparing the control group and the CYP groups. CYP mice showed significantly higher lick rates on days 3, 5, 8, and 18. Control mice showed significantly higher lick rates on day 22.

Single CYP injection mice showed significantly higher NaCl lick rates when compared to control mice on days 2, 8, and 18, as seen in Table 11 and Figures 9-11. Control mice showed significantly higher lick rates on day 22. Multiple injection mice showed significantly higher NaCl lick rates when compared to control mice on days 8, 9, and 18, as seen in Table 12 and Figures 10 and 11. Control mice showed significantly higher lick rates on day 22.

Dosage	Day	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	2	0.768	0.054	(1,29) = 6.18	**0.019**
Single		0.948	0.048		
Control	8	0.747	0.086	(1,20) = 5.57	**0.029**
Single		1.045	0.092		
Control	18	0.300	0.068	(1,8) = 10.22	**0.012**
Single		0.594	0.061		
Control	22	1.118	0.058	(1,17) = 11.41	**0.003**
Single		0.857	0.052		

Table 11: Drug state by concentration analysis comparing the control group and single CYP injection group. Single injection mice showed significantly higher lick rates on days 2, 8, and 18. Control mice showed significantly higher lick rates on day 22.

Dosage	Day	Mean Normalized licks	SEM	*F*-ratio (df_n, df_d)	*p* (Bolded if <0.05)
Control	8	0.747	0.064	(1,31) = 6.57	**0.015**
Multiple		0.957	0.052		
Control	9	0.506	0.061	(1,23) = 4.73	**0.040**
Single		0.679	0.050		
Multiple	18	0.300	0.058	(1,14) = 16.74	**0.001**
Single		0.606	0.047		
Control	22	1.118	0.062	(1,17) = 11.41	**0.003**
Multiple		0.846	0.050		

Table 12: Drug state by concentration analysis comparing the control group and multiple CYP injection group. Multiple injection mice showed significantly higher lick rates on days 8, 9, and 18. Control mice showed significantly higher lick rates on day 22.

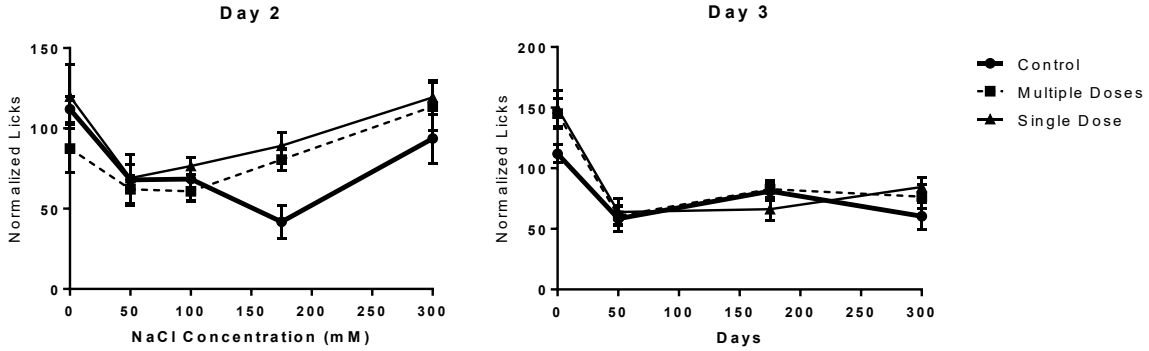

Figure 9: Normalized lick rates for days 2 and 3. Single injection mice had significantly higher lick rates compared to control mice on day 2 (p =0.019). Day 3 was included for comparison.

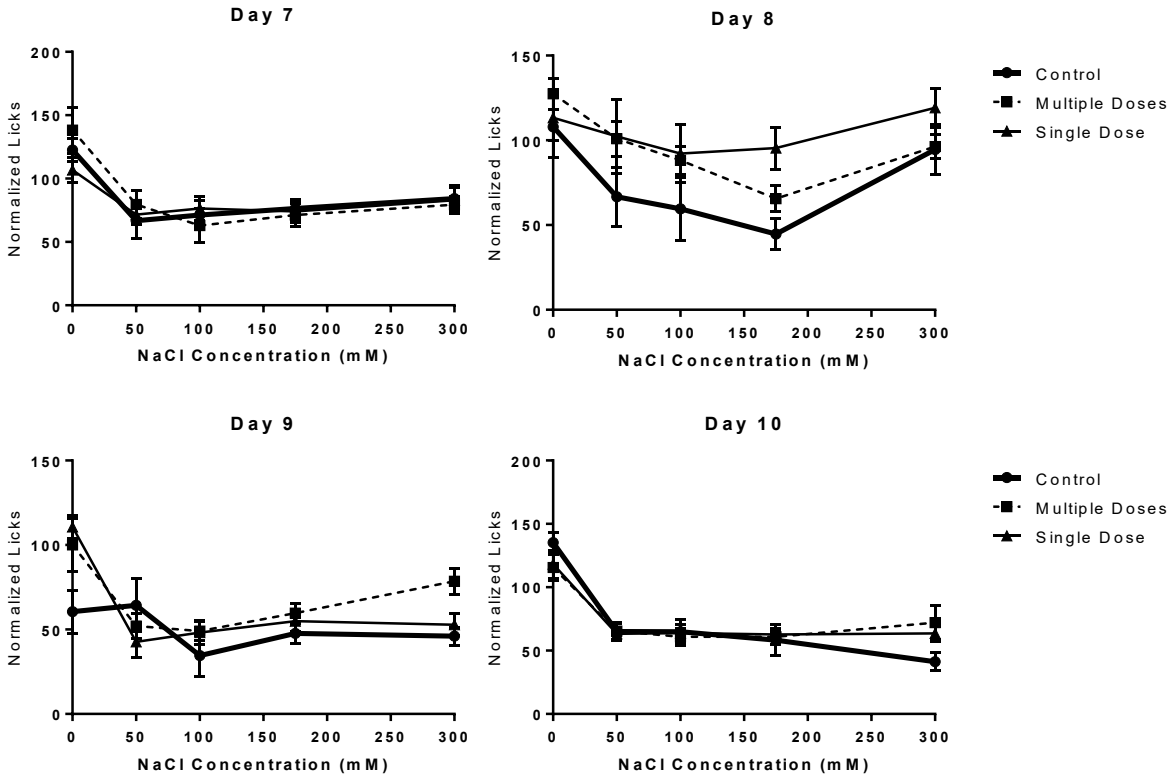

Figure 10: Normalized lick rates for days 7, 8, 9 and 10. Single injection and multiple injection mice had significantly higher lick rates compared to control mice on day 8, and multiple injection mice had significantly higher lick rates compared to control mice on day 9. Days 7 and 10 were included for comparison.

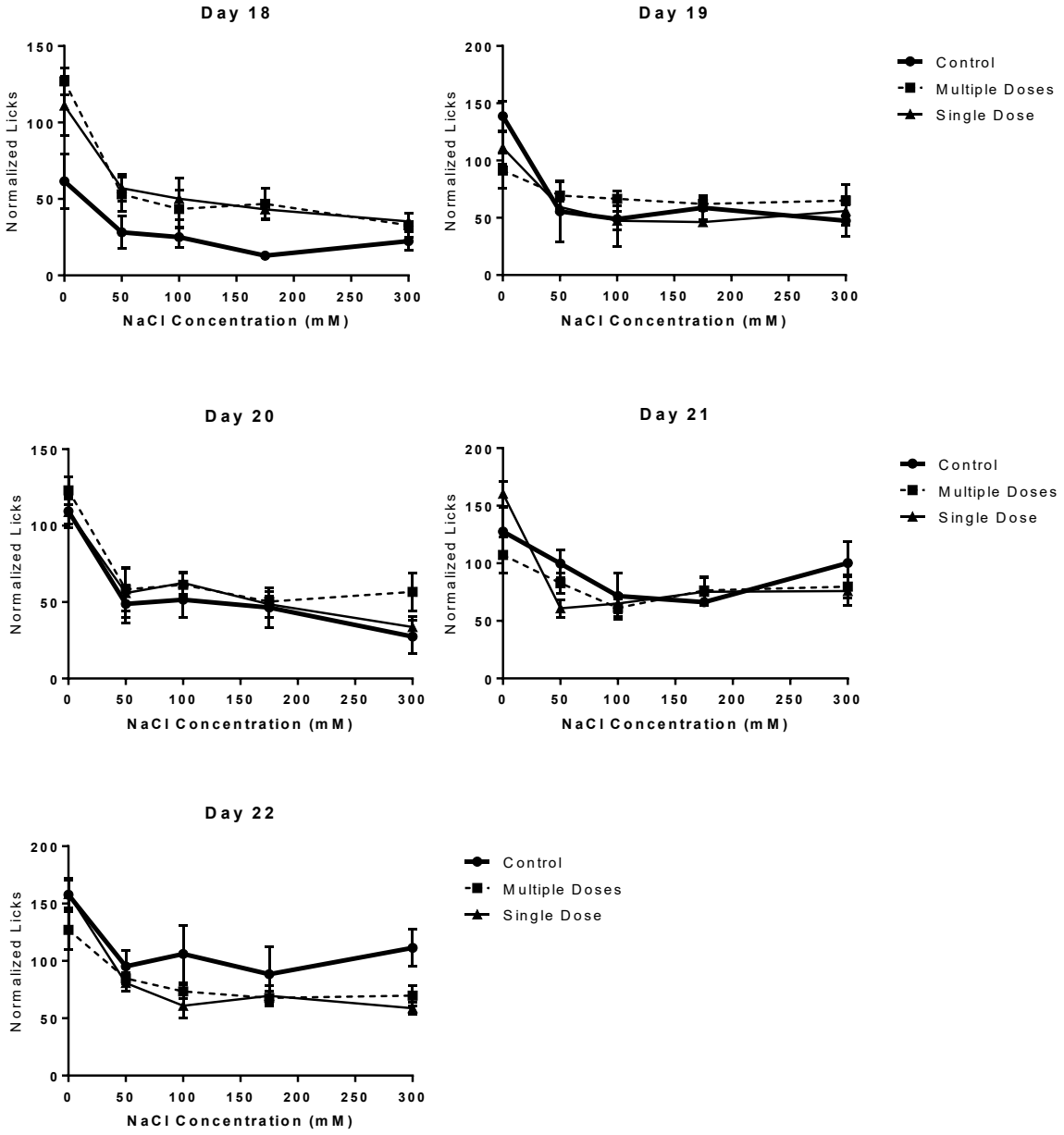

Figure 11: Normalized lick rates for days 18, 19, 20, 21, and 22. Single injection and multiple injection mice had significantly higher lick rates compared to control mice on day 18. Control mice had significantly higher lick rates on compared to multiple and single injection mice on day 22. Days 19-21 were included for comparison.

In summary, CYP mice showed significantly higher lick rates compared to control mice at 175 mM NaCl and on days 8, 9, and 18. There were no significant differences between single CYP dose and multiple CYP dose mice for any concentration.

Discussion

In this study, we attempted to determine the different effects on salt taste of a single dose of CYP compared to multiple doses of CYP spread out over five days. Our hypothesis was that CYP treatment would affect Type I taste cells, leading to changes in salt preference. We further hypothesized that multiple doses over time would affect multiple salt receptor cell types leading to more significant changes in salt preference. While we found significant CYP effects on the lick rates of NaCl, we did not see any significant difference in lick rates between single injection mice and multiple injection mice.

Since both the single and multiple injection groups of mice received an overall dosage of 100 mg/kg CYP, the lack of difference between groups could indicate that dose volume has more influence than the timing of the dose. That being said, the CYP mice showed a significantly higher lick rate compared to control mice at 175 mM NaCl. This significance, however, may not necessarily be a result of CYP. The control mean lick rate at 175 mM NaCl is lower than it is at other concentrations, including 300 mM, which was unexpected due to the natural NaCl aversion at 300 mM. Figure 8 illustrates this low lick rate for control mice not seen at other concentrations. This could be attributed to the small overall sample size for the control group, especially for days 15-31. Re-running these analyses after the present group of mice is completed may be beneficial and help to clarify these results.

All mice licked the 100 mM sucrose at rates that exceeded water, indicating that fear of new tastes (neophobia) did not influence our results. We saw significantly higher licks in both CYP groups compared to control mice on days 8 and 18. This range is fairly consistent with the proposed life span of the two different populations of Type I taste cells discussed by Perea-Martinez et al. (2013) which are eight days for one sub-population and 24 days for the second

sub-population (Perea-Martinez et al., 2013). The observed second disturbance is off from the proposed value in their paper, but our study still supports that there are two sub populations with differing life spans.

We expected to see significant group differences at 300 mM based on our CTA study, but this was absent for all comparisons made. This could be attributed to individual NaCl preference in the mice tested. The general avoidance curve we expected to see in control mice was absent for most days, and control mice seemed to prefer 300 mM NaCl.

Chapter IV: General Discussion

This paper describes our experience with two techniques to examine the effects of CYP on salt taste in mice. The first study used a conditioned taste aversion (CTA) study to test if aversion to NaCl changed after CYP treatment. The second used a simple brief access test (BAT) to compare the different effects of a single CYP injection and multiple CYP injections over five days. Generally, our hypothesis was that CYP treatment would affect Type I taste cells, leading to changes in salt preference. We further hypothesized that CYP treatment would reduce salt aversion. Lastly, we hypothesized that multiple CYP doses over time would affect multiple salt receptor cell types leading to more significant changes in salt preference.

Common Effects of CYP

In both studies, CYP treated mice showed significantly higher lick rates than control mice for at least one NaCl concentration. The drug effect was significant at more concentrations in the CTA study, while 175 mM NaCl was the only concentration where significant drug effects were seen in the second study. As stated in Chapter III, a larger sample size could be beneficial in both studies to mitigate the differences in individual mouse licking patterns. Still, the differences seen in the CTA study are enough to conclude that CYP does affect the NaCl taste receptor pathway. Since there were significantly higher licks for CYP mice than control mice in both studies, it can be assumed that CYP affects multiple salt receptor cell types. The individual level at which each is affected, however, is impossible to determine given our results. The goal of the second study was to affect the multiple pathways with multiple CYP injections over days. Since there were no differences between the single injection group and the multiple injection group, we were likely unsuccessful in affecting other salt receptor cell types with multiple injections. These results also

demonstrate that multiple injection CYP regimens do not necessarily have more severe taste related side effects than single dose CYP treatment, although the side effects may last the length of the treatment regimen.

Mice conditioned to avoid NaCl did not show significant drug effects at as many concentrations as non-conditioned mice. This could indicate that a CYP insensitive pathway remains unaffected, or affected in a different time frame. A study by Ruiz et al. indicated that while the TRPV1 receptor is not the main secondary salt taste receptor, it could be involved in some NaCl aversion (Ruiz et al., 2006). Studies have indicated that TRPV1 channels play an important role in DNA repair following radiation damage to DNA. For this reason, it would be beneficial for TRPV1 channels to be protected from damage caused by exterior agents such as CYP or radiation (Masumoto, Tsukimoto, & Kojima, 2013). These protected NaCl taste cells may be responsible for the continued aversion to low concentration NaCl following CYP treatment.

Timing of CYP Effects

In both the CTA and BAT studies, CYP mice showed significantly higher lick rates during the same time periods post-CYP injection. This range spanned from days 5-9 and days 18-19. A study by Perea-Martinez et al. (2013) proposed that Type I taste cells have two sub-populations, one with a life span of eight days and another with a life span of 24 days. The cells that have a life span of eight days are proposed to be mature cells, while the cells with a life span of 24 days are thought to be immature cells that mature and differentiae to become Type I cells (Perea-Martinez et al., 2013). Our study supports the finding of an initial eight-day life span of mature Type I cells given that this was the time period that the first NaCl taste disturbance occurred in our CYP mice. The secondary disturbance noted, which occurred around day 18,

could be the second population described as “immature Type I cells.” However, since our day 18 disturbances were several days off from the proposed second disturbance, this could indicate that a different taste cell type is being affected. Another cell type could have a different life span than Type I taste cells, and this second cell type could be responsible for the second, day 18 disturbance.

Another explanation could involve the replacement cycle for the cells. Nguyen et al. proposed that patients treated with head and neck radiation see a delayed disturbance after treatment due to the disturbance of the cell replacement cycle (Nguyen et al., 2012). After mature cells die, progenitor cells replace them. However, if the CYP is affecting progenitor cells, the dead cells are not replaced, leading to an alteration in taste later in time.

A study by Mukherjee and Delay in 2011 showed that umami taste was affected by CYP in two phases. They saw a disturbance at days 2-4 and days 9-12, which is slightly different than the biphasic disturbance that we saw when studying NaCl taste. This is likely due to the different receptors involved in salt taste and umami taste. The two phases seen in both the present study and their study suggest that biphasic taste disturbances are typical for CYP treated mice. Mukherjee and Delay also noted that fungiform papillae were most affected by CYP treatment and did not begin to recover until day 12. While we did not see the disturbance on days 2-4 that they observed, we did have residual CYP affects that persisted during the first disturbance to day 13. This could be indicative of the fungiform recovery period discussed by Mukherjee and Delay (Mukherjee & Delay, 2011).

This secondary disturbance can also be explained by an entirely different mechanism. The body is able to compensate for lost cell types by upregulating other pathways and speeding up differentiation of other cell types, as hypothesized by Ruiz et al. in their study of TRPV1

knockout mice (Ruiz et al., 2006). By disrupting Type I taste cells with CYP, it is possible that the 24-day life span of the secondary population of taste cells was sped up and these cells differentiated quicker to compensate for the cells lost during CYP treatment. This could explain the day 18-19 disturbance that we saw and explain why it did not occur on day 24 as predicted.

CTA vs. BAT study

As discussed above, the CTA study mice showed significant drug effects on more days and at more concentrations of NaCl than the BAT study mice. This difference could be attributed to differences between mouse litters involved in each study. The control animals for the BAT study did not have consistent data with the controls from the CTA study, which was unexpected due to the similar experimental conditions. An aberrant control group could have confounded our results for the second study. This problem will likely be mitigated once more mice complete the study, and a second control group is factored into the analyses.

Taste Study Criticisms

A criticism of taste studies is ensuring that animals use only the taste cue to identify the solution and its concentration. Since mice are obligatory smellers, many of their sensory cues come from the scents they perceive (Spector, 2003). While no control for scent was used in this study, the BAT paradigm is designed to minimize olfactory effects on taste since mice are presented very briefly with each stimuli (Glendinning et al., 2002). Additionally, chemotherapy treatment significantly impairs olfactory function (Steinbach et al., 2008). These factors should control for olfaction which might otherwise conflict with taste cues.

A BAT was used in this study instead of a two-bottle preference test for several reasons. Two-bottle preference tests can induce post-ingestive effects which could confound results

(Spector, 2003). We attempted to control for post-ingestive effects by providing mice with water an hour after each session to limit gastric malaise induced by overconsumption of salt. BAT studies also allow for the presentation of multiple NaCl concentrations at one time, whereas two-bottle preference tests limit each test to two solutions.

Rodents vs. Humans

The difference in salt taste receptor cells in rodents and humans is not well studied. This is likely due to the general lack of knowledge about the cell types responsible for salt taste. In terms of umami taste, humans are 70% analogous to rodents for the T1R gene, responsible for umami taste detection (Hoon et al., 1999). For salt, amiloride in humans does not appear to alter the saltiness of NaCl, but it does reduce the "sour" taste associated with it. This indicates that there are species differences in the salt taste receptors (Breslin & Spector, 2008). Further research comparing rodent and human salt receptors should elucidate these differences, which will provide further insight into mammalian salt taste.

CYP as a CTA Inducer

While our first study in Chapter II used lithium chloride to induce a CTA in mice, CYP is also an agent used to induce CTA in mice (Lin et al., 2014). We controlled for this potential confounding factor by giving the mice 24 hours of water before and after all CYP injections so that the CYP did not induce an aversion to NaCl. Additionally, the testing period (where NaCl was presented daily without CYP injection afterwards) would act as an extinction period, which would limit the effects of the potential CTA to NaCl induced by CYP. If a CTA to NaCl was induced by CYP, we would have expected to see lower lick rates for NaCl in CYP mice, which is

the opposite of what we observed. Therefore, CYP-induced CTA did not seem to confound our experiment.

Implications for Chemotherapy Patients

The reduced salt sensitivity we observed in mice matches what is reported by chemotherapy patients. Additionally, the existence of a secondary salt taste cell type which is protected from CYP has important implications. If molecular studies can pinpoint the specific cell type involved and uncover the mechanism involved in CYP resistance, this same mechanism could potentially be utilized to protect other cell types from the toxic effects of CYP.

Future Directions

A key to further uncovering the effects of CYP on salt taste is repeating the experiment with more mice under the same previous conditions. A higher number of subjects will improve our confidence about conclusions from these studies. Similar tests could also be performed involving NaCl solutions mixed with amiloride, which would eliminate effects of the amiloride-sensitive pathway and focus wholly on the yet to be agreed upon amiloride-insensitive pathway. Additionally, discrimination work from Dr. Eugene Delay's lab involving salt taste with CYP treated mice would be useful in supporting preference data from this study. Molecular studies would also be useful to uncover the other cell types that are involved in salt taste.

Conclusion

Mice show a biphasic disturbance in NaCl taste following cyclophosphamide treatment. Phase 1 of this disturbance occurs around day 8, while phase 2 occurs around day 18. Mice conditioned to avoid NaCl maintain an aversion to low concentrations of NaCl following CYP treatment, pointing to a category of NaCl receptors which are protected from the effects of CYP.

Multiple CYP injections have the same effect as a single CYP injection as long as the overall dose is the same, indicating that this is not a useful method to disturb multiple salt receptor cell types. Our results support the hypothesis that multiple taste receptor populations are involved in salt taste.

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