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2018

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Recommended Citation

Raymond, Martin, "Optogenetic examination of salt taste in mice" (2018). *Master's Theses and Doctoral Dissertations*. 928.
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Optogenetic Examination of Salt Taste in Mice

by

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Thesis

Submitted to the Department of Psychology

Eastern Michigan University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Experimental Psychology

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June 8, 2018

Ypsilanti, Michigan

Abstract

This thesis describes a series of experiments designed to evaluate the hypothesis that Type I taste receptor cells play a critical role in the detection and transduction of sodium taste via of epithelial sodium channels (ENaCs). Experiment 1 validated the function of a simple and affordable behavioral apparatus (hardware and software) for testing taste preference and taste aversion in mice. Experiment 2 demonstrated a pharmacological method for rapid induction of salt appetite in mice. Experiment 3 showed that optogenetic stimulation of Type I taste receptor cells (TRCs) in transgenic mice could drive consumption of tap water under conditions of salt hunger. The fourth and final experiment assessed whether conditioned taste aversions to sodium would generalize to optogenetic stimulation of Type I taste receptor cells in transgenic mice, with inconclusive results.

Table of Contents

List of Tables

List of Figures

**Introduction**

Humans are conventionally considered to have five exteroreceptive senses: sight, hearing, touch, taste, and smell. While vision, audition, and somatosensation are fairly well understood systems, the chemical senses (gustation and olfaction) remain somewhat more mysterious. Several potential explanations may account for this. One may be a question of interest; arguably, vision, hearing, and touch are just more relevant to our functional capacity than taste or smell and have thus received more attention. However, this sort of argument would ignore the fundamental role that these two senses play in ingestive behavior, which in turn has substantial repercussions on both physical health and quality of life. Loss of either sense can be associated with reduced appetite, weight loss, and physical complications (Schiffman, 1997) in addition to depression (Smeets et al., 2009) and increased suicidality (Joo, Hwang, Han, Seo, & Kang, 2015). Additionally, industrial research in food science targets the systems of taste and smell to generate appetitive and inexpensive food, which has in turn contributed to a variety of ingestion-related systemic health problems in our society, from hypertension (Ma, He, & MacGregor, 2015) and stroke (Polonia, Monteiro, Almeida, Silva, & Bertoquini, 2016) to diabetes (Popkin, 2015) and obesity (Vandevijvere, Chow, Hall, Umali, & Swinburn, 2015).

Clearly, we are not lacking an incentive to study taste and smell. What we lack, rather, is stimulus control. Light and sound are relatively simple stimuli to manipulate; one can accurately control the duration, wavelength, and amplitude of a light or sound generated. These stimuli can be finely tuned, and then applied and removed at the speed of light and sound, respectively. Touch is slightly more difficult to manipulate, but it is ultimately not prohibitively difficult to control the location, duration, and intensity of a tactile stimulus. The same is not the case for smell and taste. Both taste and smell exist to detect complex chemical mixtures, which by default must be dissolved and delivered in some fluid. Particularly for olfaction, this makes stimulus intensity, onset, and offset difficult to control.

Taste presents similar challenges in controlling stimulus application and removal, with some added complications. Taste is intrinsically tied to multiple other sensory systems. Traditionally, presentation of a taste stimulus dissolved in fluid necessarily provides tactile, thermal, and frequently

olfactory components as well, which have the ability to activate taste receptors independently of a taste stimulus (Cruz & Green, 2000; Lundy & Contreras, 1999; Ogawa, Sato, & Yamashita, 1968) or to modulate that activation (Breza, Curtis, & Contreras, 2006; Burseg, Camacho, Knoop, & Bult, 2010; Lemon, 2017; Shimemura, Fujita, & Kashimori, 2016). Additionally, taste stimuli could influence taste bud cells independent from specialized receptors, as many are hyperosmotic, acidic, or basic, or can actually function as neurotransmitters themselves, as is the case with many amino acids. This has made it incredibly difficult to study discreet components of the taste system, as all stimuli presented have the dual challenge of being temporally indistinct and being embedded in what is in some cases a literal torrent of extraneous noise. As a result, most investigators try to minimize tactile and thermal transients by maintaining a constant flow of solutions through the oral cavity and controlling solution temperature (Breza et al., 2006; Lemon, Kang, & Li, 2016; Lundy & Contreras, 1997). Fortunately, the inception of genetic engineering has provided a variety of new methods for addressing those problems. Light, one of the more easily controlled stimuli, is also one of the few things which will not naturally activate taste receptors, but by genetically modifying taste receptor cells to express light-sensitive ion channels, we can selectively alter their activity. This, for essentially the first time, has opened the door to fine analysis identifying the function of receptor cells and what taste qualities they signal to the brain.

That is our purpose here: While the taste-receptor cells responsible for detecting most tastes have been provisionally identified (Clapp, Yang, Stoick, Kinnamon, & Kinnamon, 2004; Kataoka et al., 2008), the taste of salt remains poorly understood. As such, we have conducted a series of experiments using an optogenetic model to examine the cellular antecedents of salt taste in mice.

**Literature Review**

As one of our five basic senses, the system of taste is critical to our experience of the world. Heterotrophs all across the spectrum of biological complexity have their survival contingent on taking foreign matter into their bodies, which is an inherently risky proposition. To begin with, many foreign substances in our environment are toxic. Ingestion of lethal substances is a constant danger to most organisms, and it becomes critical to have some system in place to identify those substances before it's too late. A variety of senses are helpful in this capacity; vision and olfaction allow us to identify and avoid things in our environment that we know to be poisonous. Some toxic organisms subject to being ingested have even developed their own visual cues to assist us in making that discrimination. However, these systems of detection are imperfect. Visual cues are not always present, and when present, they are not always reliable. Furthermore, most external cues of toxicity rely on previous experience with that particular cue, which is less than ideal when trying to avoid a lethal substance. Overall, it is beneficial to have a system in place to not merely identify potential sources of toxins but to specifically detect the toxins themselves.

Additional considerations ought to be made; the percentage of substances in our environment that are toxic are far outweighed by substances that merely lack nutritional value, either universally or in the context of a specific need state. As a result, it becomes imperative to detect useful and necessary chemicals in addition to the detection of harmful substances. This is where taste comes into play. While vision and smell allow us to act on our learned experience of nutrition in the environment, taste acts as a gatekeeper, detecting toxic or nutritional substances directly, to allow us to reject or ingest them respectively. This system can be informed by learning (Garcia, Kimeldorf, & Koelling, 1955) but does not depend on it (Grill & Norgren, 1978); from the first day we are born, humans are programmed to reject substances that taste bitter and to ingest substances that taste sweet (Steiner, 1973; Steiner, Glaser, Hawilo, & Berridge, 2001). This has made taste critical to our survival whenever and wherever food is scarce. All of those implications shift in situations where food is readily available and ingested toxins are relatively rare. In modern societies, while hunger remains an issue, issues of overconsumption have also

become problematic. When highly palatable food is highly available, the natural survival mechanism motivating us to ingest sweet and salty foods can endanger our health. In American society alone, overconsumption of highly caloric foods is associated with obesity (Ludwig et al., 1999; Ruhm, 2012), a condition associated with a projected cost of $190 billion annually in medical expenses (Cawley & Meyerhoefer, 2012). Overconsumption of salty foods is associated with hypertension and stroke, which exact staggering medical costs, potentially as much as $800 billion by 2030 (Heidenreich et al., 2011). A mere 10% reduction in sodium intake would be projected to save $83 million in annual healthcare costs (Webb et al., 2017). As such, it becomes critically important for us to understand the taste system that so impacts our own health, so that by better understanding it we may be able to modify its function to better suit our needs.

Any understanding of taste must necessarily begin with the tongue. Not only does it contain the vast majority of the chemoreceptors associated with our sense of taste as we traditionally understand it, but the physical morphology of the tongue also has substantial implications in taste. This morphology is represented in the form of papillae, the multitude of small protruding structures on the tongue. Four types of taste papillae have been characterized: fungiform, foliate, circumvallate, and filiform papillae. The overwhelming majority of papillae on the tongue are the filiform (Hume & Potten, 1976; Iwasaki, Okumura, & Kumakura, 1999), which contain no taste receptors (Mbiene, Maccallum, & Mistretta, 1997). However, they are thought to serve a number of other functions; they serve as important structural components of the tongue (Wong et al., 2000) and as tactile receptors (Suemune et al., 1992). As such, these papillae do serve a critical role in feeding but are not themselves receptive to taste.

The remaining papillae all contain taste buds. Commonly confused for papillae, taste buds are clusters of taste receptor cells embedded within the papillae. A single papilla can contain one or more buds. Fungiform papillae in mice and rats typically contain a single taste bud (Venkatesan, Boggs, & Liu, 2016), whereas in humans they can contain multiple buds (Arvidson & Friberg, 1980). These fungiform papillae are clustered on the dorsal surface of the anterior two thirds of the tongue, and as such, they are innervated by the chorda tympani (Oakley, 1975), a branch of the facial nerve (Cranial Nerve [CN] VII).

They serve as a highly important structure tied to taste discrimination (Spector, Markison, St. John, & Garcea, 1997), particularly salt-sensing (Slotnick, Sheelar, & Rentmeister-Bryant, 1991; St. John & Spector, 1998). Like the filiform papillae, fungiform papillae likely serve an important function for somatic sensation in the oral cavity as well (Suemune et al., 1992).

Circumvallate papillae are clustered medially on the posterior tongue. Humans tend to have many circumvallate papillae, but mice have only one (Jung, Akita, & Kim, 2004). This provides an interesting contrast to their fungiform papillae; while they have many fungiform papillae that each contain one taste bud, mice only have a single circumvallate papilla, which contains over a hundred taste buds (Miller & Whitney, 1989). The taste buds of the circumvallate papilla are innervated by the glossopharyngeal nerve (CN IX).

Finally, the foliate papillae are situated along the lateral surface of the posterior of the tongue. While less numerous than the fungiform papillae, the foliate papillae do contain multiple taste buds (Venkatesan et al., 2016). Like the circumvallate, the taste buds of the foliate papillae are innervated by CN IX. In rodents the response profiles of the glossopharyngeal nerve, which innervates these papillae, are quite different than those associated with the fungiform papillae. Specifically, the glossopharyngeal nerve is only weakly responsive to sodium salts, and has substantially more specialized responses to bitter stimuli (Frank, 1991). As a result, it seems likely that the foliate and circumvallate papillae serve a different function from that of the fungiform papillae, potentially as a warning mechanism against toxic substances.

Additionally, the taste receptor cells in a bud are centered around a taste pore, a feature of the papillae that establishes contact between the sensory surface of the taste system and the external environment. The structure of this pore alone may be significantly related to global taste function (Whiddon, Rynberg, Mast, & Breza, 2018), as not all papillae possess an obvious pore, and the proportion that lack a pore seems to increase with age. Interestingly, in early development, taste receptors in pore-lacking papillae remain functional, as portions of the papillar tissue are permeable (Mbiene & Farbman, 1993).

Taste bud cells have been categorized into three main varieties: Types I, II, and III. These cells are not entirely understood in the totality of their function, though some types and subtypes have been more thoroughly described than others. Type II cells are perhaps the most clearly understood; subtypes of cells in this category are known to respond preferentially to sweet, bitter, or umami stimuli, with essentially little to no overlap in taste quality functionality. These response patterns are determined by the membrane protein that acts as a receptor; sweet stimuli are detected by a combination of the T1R2 and T1R3 receptors (Li et al., 2002; Nelson et al., 2001), while umami stimuli are primarily detected by the T1R1 and T1R3 receptors (Li et al., 2002; Nelson et al., 2002), though perhaps not entirely (Blonde, Travers, & Spector, 2018). Bitter stimuli are detected by a slate of varying T2R receptors (Chandrashekar et al., 2000), reflecting the importance of detecting a wide variety of potentially toxic substances. It has been proposed that information is communicated to the afferent nerve fiber without a traditional synapse, via release of ATP (Y. J. Huang et al., 2007), since Type II cells lack traditional synapses (Chaudhari & Roper, 2010).

The function of Type III cells has been identified somewhat less definitively. On one hand, they are the only receptor in the taste system known to have a synapse with their afferent nerve fiber (Murray, 1993; Yee, Yang, Bottger, Finger, & Kinnamon, 2001) and release serotonin and norepinephrine (Y. A. Huang, Maruyama, & Roper, 2008). On the other, their best stimulus remains somewhat unclear. They are known to respond to sour tastes (Y. A. Huang, Maruyama, Stimac, & Roper, 2008) and acids via a polycystic kidney disease-like ion channel (PKD2L1) (Horio et al., 2011), but elimination of these channels does not totally abolish sour taste. Targeted ablation of PKD2L1 cells, however, results in complete loss of sour taste (A. L. Huang et al., 2006), indicating that Type III cells that express PKD2L1 channels are necessary for sour taste. They may also play a role in detecting salt stimuli through an amiloride (a potassium-sparing diuretic and epithelial sodium channel blocker) insensitive pathway (Lewandowski, Sukumaran, Margolskee, & Bachmanov, 2016).

The least understood of the taste receptor cell types is the Type I cell. These cells have been hypothesized to serve purely supportive, glial-like function (Dvoryanchikov, Sinclair, Perea-Martinez,

Wang, & Chaudhari, 2009), but that has not been firmly established. On the other hand, there is some evidence that they may express epithelial sodium channels (ENaC) (Vandenbeuch, Clapp, & Kinnamon, 2008), a membrane protein well understood to play a substantial role in detecting sodium salts (Heck, Mierson, & DeSimone, 1984). As GAD 65 (glutamic acid decarboxylase, an enzyme that breaks down glutimate into GABA) expressing cells, they are also known to release the neurotransmitter GABA (Dvoryanchikov, Huang, Barro-Soria, Chaudhari, & Roper, 2011) and are hypothesized to release ATP (Houser, Breza, Balasubramanian, Travers, & Travers, 2017), which can serve as a neurotransmitter as well (Burnstock, 1972; Finger et al., 2005).

Further complicating matters, the receptor type or types responsible for detecting nonsodium salts independent of the ENaC pathway has not been identified. It may be that the Type I cells serve this function, or the Type III cells. It could be that these two receptor types identify different kinds of salts or work together to discriminate salts similar to the function of cones in the retina.

However varied these cell types are in their receptive function, they share in common their pathways for communication with the brain. All taste information from the tongue is communicated to the brain via a combination of the facial and glossopharyngeal cranial nerves. The facial nerve contacts the anterior two thirds of the tongue and innervates the taste receptors there, while the glossopharyngeal nerve innervates the posterior third of the tongue. Additionally, the vagus nerve communicates some taste-like chemoreception signals from the epiglottis and gut (Contreras, Beckstead, & Norgren, 1982; Contreras, Gomez, & Norgren, 1980).

While the facial nerve (CN VII) carries considerably more information than its chemosensory component, the taste information communicated by CN VII is largely segregated to a subdivision of the nerve known as the chorda tympani (CT). The nerve fibers of the chorda tympani begin on the basal surface of the taste buds of the anterior two thirds of the tongue (Oakley, 1975) and from there run down the length of the tongue to the geniculate ganglion, before synapsing in the rostral portion of the nucleus of the solitary tract (NTS) in the medulla (Contreras et al., 1980). Taste responses in the chorda tympani have been studied extensively, allowing for a reconstruction of various neural response profiles to taste

stimuli. This study of taste response profiles has resulted in the identification of a number of stereotyped categories for taste-responsive neurons, breaking down along the lines of the taste stimuli they respond best to. Classically, there are clusters of cells that respond best to sucrose (sweet), sodium chloride (salty), hydrochloric acid (sour), or quinine hydrochloride (bitter) (Frank, 1974).

We are particularly interested in the neurons that respond best to salts. Based on single cell and behavioral data with amiloride, our working hypothesis is that Type I taste bud cells transmit sodium ($Na^+$) taste to NaCl-best neurons via ENaCs, whereas Type III taste bud cells transmit sodium taste to acid-generalist neurons via an unknown receptor mechanism. The pertinence of salt-sensing cells in salt taste is fairly obvious, but sour-sensing cells also have a very important role to play. While the vast majority of the response of salt-best neurons is amiloride-sensitive, the sour-best neurons have also been found to respond to salts, a response which is largely insensitive to amiloride (Hettinger & Frank, 1990). This suggests multiple neural pathways for salt-taste transduction, a finding that invites further study into the intricacies of salt detection. Neurons that respond preferentially to sodium salts (NaCl-best neurons) are known to communicate with ENaC-expressing taste-bud cells, as their responses to sodium salts are greatly diminished by amiloride and amiloride analogs (Breza & Contreras, 2012a, 2012b; Breza, Nikonov, & Contreras, 2010; Lundy & Contreras, 1999; Ninomiya & Funakoshi, 1988; Rehnberg, MacKinnon, Hettinger, & Frank, 1993). In contrast, neurons that respond broadly to both salts and acids (acid-generalists or electrolyte generalists) are  not affected by amiloride (Lundy & Contreras, 1999; Ninomiya & Funakoshi, 1988; Rehnberg et al., 1993) or amiloride analogs (Breza & Contreras, 2012a, 2012b; Breza et al., 2010), suggesting they are in contact with taste-bud cells that express a different salt-taste receptor. While the CT is often over-generalized as a taste-specific nerve, it is also know to transmit tactile (Finger et al., 2005) and thermal information (Breza et al., 2006; Lundy & Contreras, 1999; Ogawa et al., 1968). In addition to the CT, an additional subdivision of the facial nerve, the greater superficial petrosal nerve (GSP), which innervates the soft palate and nasoincisor duct in rodents, is also known to transmit salt taste information through amiloride-sensitive and amiloride-insensitive mechanisms (Dinkins & Travers, 1998; Nejad, 1986; Sollars & Hill, 1998). This may be a particularly pertinent

pathway to salt taste transduction in some animals, as even animals that demonstrate no amiloride-sensitive salt response in the CT have yet displayed amiloride-sensitive detection of salts in their behavior, indicating a CT-independent pathway (Eylam & Spector, 2005). This is important because amiloride functions by blocking ENaC in both the kidneys and the tongue (Heck et al., 1984), thus inhibiting the major sodium-detecting receptor protein.

**Behavior**

A great deal of investigation of the taste system is conducted using electrophysiology and recording from either individual cells in the brain or whole nerves. However, while these electrophysiological techniques allow a great deal of experimental control with which to study taste system function, the behavioral and perceptual correlates and implications of physiological findings are not always clear. Often, it is useful or even necessary to collect information from the behavior of animals as well, whether they are human or rodent.

Very different techniques tend to prevail in human and rodent models, for obvious reasons. While the ability of humans to communicate verbally is very useful for sensory discrimination tasks (Hettinger, Gent, Marks, & Frank, 1999) and threshold testing (Bartoshuk, Gent, Catalanotto, & Goodspeed, 1983), ethical considerations preclude the use of some more robust and specific behavioral models. For instance, it is generally impossible to eliminate the function of a cranial nerve in a human subject, though special anesthetic procedures do permit this in some cases (Lehman, Bartoshuk, Catalanotto, Kveton, & Lowlicht, 1995). In a rodent, one can sever the nerve in question.

This is reflected in a variety of other interventions used to study behavior in rodents; consumption is a fairly direct indicator of taste perception, and consumption can be manipulated. Commonly, some state of physiological need is created by limiting access to water or specific nutrients or using pharmacology to make some needed substance physiologically unavailable. Water deprivation can be achieved by restricting access to water or pharmacologically by injection of polyethylene glycol (Stricker, Gannon, & Smith, 1992). Hyponutremia can be accomplished similarly; salt depletion can be induced via dietary sodium restriction (Contreras, 1977; Prakash & Norgren, 1991) or by the use of diuretics that

prevent retention of sodium (Caloiero & Lundy, 2004). These interventions in turn drive consumption under controlled conditions; water deprivation causes animals to drink water, which is highly effective in forcing consumption of normally noxious stimuli (Spector & St. John, 1998), or simply condensing consumption into a more easily studied window.  Similarly, depriving an animal of sodium motivates that animal to consume sodium in quantities and at concentrations typically avoided (Berridge, Flynn, Schulkin, & Grill, 1984).

Alternatively, interventions can be made to induce avoidance of stimuli, even those normally preferred. The classic example of this paradigm is conditioned taste aversion, where presentation of some taste stimulus is paired with a treatment that sickens the subject (Garcia et al., 1955). Thereafter, that taste stimulus will tend to be avoided when possible. The combination of this technique and need inducement allows us to study stimulus quality directly; two stimuli may elicit similar neural responses, but if an aversion conditioned to one stimulus also causes avoidance of the other, then it can be concluded that the two stimuli have a comparable quality. Unfortunately, establishing that the quality of two substances is comparable does not guarantee that they are identical. For instance, conditioned taste aversion studies indicate that a combination of monosodium glutamate and amiloride produces a taste comparable to sucrose and a variety of other sweet stimuli (Heyer, Taylor-Burds, Tran, & Delay, 2003), but animals are nevertheless capable of discriminating between this mixture and the same sweet stimuli (Heyer, Taylor-Burds, Mitzelfelt, & Delay, 2004).

**Genetic Modification**

While we have been able to establish equivalence or discrepancy of stimulus quality across different stimuli, it has long been a challenge to tie stimulus quality to physiological or anatomical structures. Historically some success in understanding the anatomy of taste sensation has been achieved by dissecting various nerve fibers and measuring the resultant effect on behavior (Frankmann, Sollars, & Bernstein, 1996; St. John, Garcea, & Spector, 1994; Vigorito, Sclafani, & Jacquin, 1987), but attempting to increase the granularity of our understanding is tied with increasing difficulties. While we have long been aware that stimulation of the taste buds results in patterned neural responses that correspond in a

stereotyped fashion to understood stimuli (Breza et al., 2006; Nakamura & Norgren, 1993; Nishijo & Norgren, 1997), we have been unable to identify the cellular mechanisms of that discrimination until fairly recently. The introduction of genetic engineering as a tool of science revolutionized the study of taste. Rather than stimulating the tongue with various chemicals that could act on an indeterminate number of receptors and pathways, genetic engineering has given us the ability to stimulate individual cell types specifically (Zemelman, Lee, Ng, & Miesenböck, 2002), or even observe the activation of specific cells directly (Nakai, Ohkura, & Imoto, 2001). This has revolutionized the study of taste, serving as the basis for identifying the function of specific taste receptor cells (TRCs). Generally, this has taken the form of designer receptors exclusively activated by designer drugs (DREADD) or receptors activated solely by a synthetic ligand (RASSL; Coward et al., 1998), where taste receptor cells are genetically modified to express a receptor that can only be activated by an otherwise tasteless drug (Mueller et al., 2005; Zhao et al., 2003).

Increasingly, taste researchers are also looking to optogenetic tools, a set of genetic modifications that cause cells to express light-activated ion channels or light-emitting proteins, or both, all under the control of specific genetic promoters. This allows researchers to stimulate specific cell types with light, an indisputably tasteless stimulus, or observe exactly which cells respond to a traditional chemical stimulus. These techniques have enabled unprecedented opportunities to examine individual receptor function, and even the organization of taste in the brain (Fletcher, Ogg, Lu, Ogg, & Boughter, 2017). Unfortunately, the integration of optogenetic and behavioral techniques is still in its infancy. While some physiological studies have directly assessed the impact of light stimulation on the tongue, behavioral studies typically involve stimulation of the brain (Peng et al., 2015; Zocchi, Wennemuth, & Oka, 2017), often based on theories that have been refuted (Fletcher et al., 2017; Mast, Breza, & Contreras, 2017). Virtually no studies have been published assessing the impact of optogenetic stimulation of TRCs on animal behavior relative to primary tastes.

**Lickometery**

In studying ingestive behavior, there are certain indispensable tools that scientists rely on. For obvious reasons, it is vital to reliably quantify the consumption of taste solutions, and a huge variety of mechanisms have been developed to address this need, from simple to complex. In the study of rodents, many of these tools focus on recording the licking of some fluid delivery spout; in general, these devices are known as lickometers.

The literature surrounding the development and use of lickometers is itself vast and arcane. The earliest documentation of a device recognizable as a lickometer extends as far back as 1951, with the device dubbed an "Electronic Drinkometer" (Hill & Stellar, 1951). While this was the first lickometer in the modern sense, it was labeled a "drinkometer" in reference to an even older class of mechanisms designed to measure the volume of fluid an animal consumes. This addressed a fairly pressing need in taste research, as measurements of fluid quantity can be highly problematic. Temporal resolution of drinking measures are quite poor; most systems for measuring volume return only a single value per behavioral trial. Methods that provide improved temporal resolution sacrifice resolution in volume, and require intensive monitoring (Hill & Stellar, 1951). Even single measurements of fluid consumed per session are relatively error-prone, considering that mice drink an average of 5.8 ml per day (Gannon, Smith, Henderson, & Hendrick, 1992), and substantially less in a 20–30 minute session. The most reliable way to quantify volume consumed is to control it directly, which then interferes with the natural behavior of the animal (Hill & Stellar, 1951; Weijnen, 1989).

One might assume that all lickometers are therefore drinkometers. However, the avenue of methods research that invention gave birth to has expanded continuously over the past 66 years, in the process revealing that the prototype lickometer fundamentally failed to achieve the desired goal; licking, as it turns out, does not reliably predict fluid volume consumed (Weijnen, 1989). On the other hand, all of that study has also established a value in measuring licks beyond an attempt to estimate drinking. A tremendous amount of work has been done to characterize patterns in licking that can elucidate the psychophysical processes of appetite and ingestion with far greater nuance than gross measurements of

fluid quantity. As such, lickometers can serve a valuable role as a companion or alternative to volume measurement.

Over time, there been numerous evolutions in the physical construction of lickometers. The Electronic Drinkometer was the first such device, and many designs retain the basic function of the original apparatus; by licking a spout, the mouse closes an electric circuit which in turn registers a lick (Davis, 1961; Dole, Ho, & Gentry, 1983; Hayar, Bryant, Boughter, & Heck, 2006). However, that design is not ideally suited to all purposes, and many different versions of the lickometer have been developed that rely on very different principles. Some rely on the disruption of a light beam (Hu, Lai, Shyu, & Tung, 1998; Schoenbaum, Garmon, & Setlow, 2001), while others rely on force transduction to generate a signal, either by measuring the disturbance of the drinking spout (Ossenkopp, Cooley, & Vanderwolf, 1980), or by forgoing the traditional spout and delivering solutions on the surface of a ball, the rotations of which are used to quantify licking (Spector et al., 2015).

Each technique for recording licks has strengths and weaknesses. Typically, the electronic lickometer's weaknesses stem from its metal components and the electrical current they pass; metal spouts and grounding components make electronic lickometers incompatible with microwave irradiation of the testing chamber. The electric signal of the lickometer itself can interfere with neural recording or, in some cases, risk detection by the animal itself if the current is strong enough to be felt. Most designs overcome at least one of these issues; modern lickometers limit their signal below the 5 µA current detectable by rats (Weijnen, 1989), and some recent designs further limit that signal to the extent of eliminating neural artifacts (Hayar et al., 2006). There are trade-offs involved; the weaker the signal of the lickometer, the more difficult it is to discriminate genuine licks from nose-pokes and other extraneous contacts (Hayar et al., 2006; Raymond, Mast, & Breza, 2018). Electronic lickometers do have many advantages as well. As the change in current that electronic lickometers record results from direct contact with the drinking spout, electronic lickometers are capable of capturing the exact moment of contact and release of contact for each individual lick, allowing for certainty that every event recorded constitutes actual contact with the spout, and further permitting the exact duration of that contact to be established. Additionally,

electronic lickometers are elegant in their simplicity; provided a system for recording electrical current, the apparatus itself requires only a metal spout and a metal ground. This allows for a great deal of adaptability and design customization, to the extent that electronic lickometers can be easily added to an existing cage (Dole et al., 1983).

Photobeam lickometers have a different set of strengths and weaknesses; they do not pass a current through the animal, making them undetectable by the animals and eliminating the possibility of electrical artifacts in brain recordings. As they do not rely on metal to form a circuit in the testing chamber, they are also compatible with microwaves. They do have limitations; since photobeam lickometers are based on the breaking of a beam of light very close to the drinking spout, the events they record imply contact and drinking, but they do not record contacts directly, making it less certain that any given activation represents a genuine contact of the mouse with the spout. This also makes licking microstructure more difficult to ascertain. While it is possible to construct a photobeam lickometer independently (Schoenbaum et al., 2001), it is substantially more difficult than building an electronic apparatus (which can ultimately boil down to some light soldering).

Force transducing lickometers carry all of the benefits of photobeam lickometers, as well as some of the perks of electronic designs. Here, rather than recording contact with the spout electrically or by the breaking of a proximal light beam, drinking events are recorded whenever the animal moves a physical mechanism. Some designs are analogous to a ballpoint pen (Spector et al., 2015), where fluid is delivered on a rolling ball, and the movement of that ball by the animal's tongue is directly measured. Others mimic an elecronic lickometer in form, but rather than recording current changes associated with making and breaking a circuit, the apparatus measures current changes associated with the physical movements of a drinking spout licked by the animal (Ossenkopp et al., 1980). Either design overcomes some of the basic potential concerns of electronic lickometers; they pass no current and require no metal in the testing chamber, making them undetectable, artifact-free, and microwave-compatible. Additionally, as they do measure contact with a drinking spout, they serve as a higher-fidelity measure than photobeam lickometers. Their weaknesses are mainly related to accessibility; these designs are much more physically

complex than electronic lickometers, making them much more difficult to implement independently. Additionally, in the case of the force-ball lickometer, the design may be incompatible with some behavioral optogenetic tests; the ball itself would interfere with any integration of light stimulation in the drinking apparatus, though not with optrodes implanted in the animal. It was our interest in combining optogenetic manipulation with conventional behavioral assays that motivated the design of the lickometer apparatus described below.

**Hypothesis**

We were particularly interested in two substantive gaps in the understanding of taste at the receptor level: the lack of a known receptor cell for salt taste, and the lack of a known taste stimulus for Type I taste receptor cells. It was our hypothesis that the Type I TRCs play a substantive role in the transduction of salt taste, explaining both missing pieces of information simultaneously. We reasoned that sodium appetite and sodium aversion would serve as reliable indicators of behavior driven by the sensation of sodium salts and that simulating this behavior by selective optogenetic stimulation of the Type I TRCs would provide evidence of salt-taste transduction by the Type I TRCs.

**General Methods**

**Subjects**

Three genetic strains of mice were used in this series of experiments: C57BL/6J mice (B6); Ai32 mice, a genetically modified C57BL/6J strain with the genotype for CRE-dependent expression of Channel Rhodopsin II and Enhanced Yellow Fluorescent Protein; and GAD65CRE/Ai32 (ChR2) Crossed mice, a genetically modified C57BL/6J strain with phenotypic expression of ChR2 and EYFP liberated exclusively on GAD65 expressing cells.

**Housing**

Mice were group-housed according to sex in standard plastic shoebox cages (28 x 17.5 x 13 cm) prior to testing. Unless otherwise noted, animals had ad-libitim access to food (LabDiet #5015) and tap water. Mice were kept on a reverse dark-light cycle (lights on from 7 pm to 7 am), and were tested during their dark cycle exclusively.

**Testing Chamber**

All behavioral tests were conducted in an acrylic chamber (Figures 1 & 2) measuring 13.5 x 11.5 x 12.5 cm. Two ports milled in the 11.5 cm side of the chamber were spaced 5 cm apart, 2 cm above the chamber floor. The chamber was floored with a tin plate. Water bottles were constructed from standard steel drinking spouts (8 mm diameter) joined to 15 ml centrifuge tubes by silicone stoppers. The lickometer circuit was routed through a 3.5 mm stereo microphone input on a desktop computer, where the left and right leads were connected to two separate drinking bottles via alligator clips, and the ground pin was connected to the chamber's tin floor, also by alligator clip. The circuit supplies 2.8 V DC, which can be reduced by the addition of resistors.

**Solutions**

Though some previous behavioral research has used deionized water as the solvent for mixing taste solutions, we mixed all taste solutions in tap water, as our animals have never had access to deionized water, and because deionized water is bitter to rodents (Grobe & Spector, 2008; Loney, Blonde, Eckel, & Spector, 2012).

**Experiment 1: An Open-Source Lickometer**

**Purpose**

Our first aim was to use readily (and cheaply) available materials and open-source software to construct and validate an affordable and reliable alternative to commercially available lickometer hardware, which can be prohibitively expensive.

**Subjects**

Subjects were six adult B6 mice, including three male (36.3 ± 3.9 g) and three female (32.1 ± 3.4 g) mice. Mice had previous experience with the experimental apparatus.

**Procedure**

Twenty-four hours prior to testing, water was removed from the home cages, though free access to chow remained. Animals were tested on two subsequent days and received all of their fluid during the test sessions. Water was replaced on the home cage at the end of testing on the second day. Lickometer recordings were initially captured with 10 MΩ resistors added to the recording circuit, and the experiment was repeated 20 days later with the additional resistance removed.

In the 30 minute experimental trials, animals were given simultaneous free access to two drinking spouts, one containing tap water and the other containing a 0.2 *M* sucrose solution. Bottles were reversed on the second day of testing to account for the established phenomenon of side preference (Bachmanov, Reed, Beauchamp, & Tordoff, 2002).

**Data Analysis**

Data were exported from Audacity (an open-source audio recording program) to R (an open-source statistical analysis program), where it was analyzed by our custom script. The script operates by setting a threshold at -3 standard deviations and recording time codes for electrical disturbances that cross that threshold. Thresholds were set manually for one session, in which an intermittent fault in the path to ground increased background noise. By default, the program removes events occurring within 40 ms of previous events, per established practice (John D. Boughter, personal communication, March 31, 2017).

This cutoff is variable, accommodating the variable lick rate of different mouse strains (Boughter et al., 2012; Glatt, St. John, Lu, & Boughter, 2016).

For comparison, we also exported our files to Spike2, a professional waveform analysis software package (Cambridge Electronic Design [CED], Cambridge, England) that has been demonstrated as an effective lick-counting program (Hayar et al., 2006). R was then used to conduct statistical analysis of the results.

**Results**

To validate the results of the R script against Spike2 in assessing the lickometer recordings, t-tests and correlation coefficients were calculated comparing licks counted on water, licks counted on sucrose, and the preference score for sucrose versus water. No significant differences were found on any of the three measures, and the scores for R and Spike2 were found to be highly correlated on all measures. Results are recorded in Table 1 and illustrated in Figure 3. Additionally, interlick intervals (ILIs, the interval in milliseconds between licks) were calculated for all licks, and distributions thereof (Figure 4) are consistent with previous lickometry findings, from the positioning of the main distribution around ~110 ms to the small secondary distribution at around twice that interval (Glatt et al., 2016). An additional small distribution of licks near 50 ms in the 10 MΩ condition is presumably a reflection of signal fidelity; increasing the resistance in the recording circuit dramatically reduced the amplitude of the signal relative to electrical noise (Figure 5), resulting in the counting of several false "double contacts." Importantly, these instances of double contact can be automatically removed, as is the standard practice in the field. We chose to leave them in this case, both in order to maintain adherence to our 40 ms cutoff and to illustrate the potential problems with the excess noise associated with high-resistance recordings. The analysis program allows for the selection of different thresholds, per user requirements.

Additionally, several tests were performed assessing the impact of electrical resistance in the recording equipment. Results are shown in Table 2, and illustrated in Figure 6. Mean primary interlick interval (MPI) describes a characteristic ILI by calculating the "mean of all ILIs less than or equal to 160

ms" (Glatt et al., 2016). Essentially, this summarizes an animal's average rate of licking, excluding long pauses.

Only a single significant difference was found, which was somewhat surprising given the severe degradation of signal fidelity occurring at 10 MΩ of added resistance. It's unclear why the lick count for sucrose increased when resistance was removed; clearly, it is not the result of aversive current, so we speculate that the shift may be due to increasing familiarity with the mechanism, particularly given that there was no change in MPI.

Finally, we ran a few tests comparing sucrose and water consumption (Table 3, Figure 7). Indices of preference for sucrose over tap water were observed consistent with expectations. Sex differences in preference score were also tested, and found non-significant ($p = 0.34$).

## Experiment 2: The Rapid Induction of Salt Appetite in Mice

**Purpose**

Our second aim was to replicate and expand upon previous findings demonstrating induction of sodium appetite by self-administration of the potassium-sparing diuretic amiloride (Caloiero & Lundy, 2004). Where the previous study had been conducted in rats and had used amiloride in conjunction with sodium-deplete diet, we aimed to assess whether the drug could also be used in mice, without altering diet. Additionally, we attempted to create an inactive control treatment for amiloride by destroying the drug with light.

**Subjects**

A total of 18 naive B6 mice were used in this study, including 8 females (26.5 ± 0.96 g) and 10 males (37.8 ± 1.93 g). All animals were C57BL/6J mice ($\bar{x}$ = 156 days). Mice were divided evenly into three groups: treatment with standard amiloride, treatment with photo-bleached amiloride, or no treatment.

**Amiloride**

Powdered amiloride was dissolved in tap water to a concentration of 300 μM, and half of this solution was set aside to be used directly. In an attempt to create an inactivated analog for the drug (since it is recommended to protect amiloride from light), the remainder of the solution was placed in a clear glass flask and exposed directly to a 40 W incandescent lamp. Total exposure time amounted to approximately 72 hrs. The two amiloride solutions were then placed in the home-cage water bottles of the mice in the experimental groups.

**Procedure**

Approximately 36 hrs prior to testing, water bottles were changed on the home cage (tap water, water + 300 μ*M* amiloride, or water + 300 μ*M* photo-bleached amiloride). Access to food (LabDiet #5015) and water were not restricted. At the time of testing, animals were removed from the home cage and placed within a custom-built lickometer apparatus. Inside the chamber, animals had 30 min of free access to two solutions; 0.3 *M* NaCl and tap water. Licking data on both spouts were collected by the

apparatus, and recorded in the open-source audio program Audacity. Each trial was repeated on the following day with the bottles reversed to account for any inherent side preference.

**Data Analysis**

Raw data recordings were exported from Audacity as .CSV files (Spike2) and imported into R (an open-source statistical software package). These data were processed via a custom script written to accompany the apparatus, calculating total lick counts on both spouts, as well as MPI, lick efficiency (% of ILIs under 160 ms), frequency and duration of lick bursts (clusters of at least three licks with ILIs not greater than 1 s) (Glatt et al., 2016), and frequency and duration of pauses between bursts. Once this information had been computed, R was also used to conduct statistical analysis of the data. Statistical tests were comprised primarily of contrast analyses when parametric tests were permissible and Kruskal-Wallis tests with subsequent pairwise Wilcox tests when data were not normally distributed.

**Results**

In order to evaluate the effect of self-administration of amiloride on sodium appetite, a number of contrast tests were conducted. Tests initially compared the two amiloride conditions to the water control condition, revealing a number of significant differences on various licking measurements, from macrostructural features like preference score and total licks to either saline or water, to microstructural measures such as burst duration (measured as the number of licks in a "burst," or a series of licks with a delay of no more than one second between any two licks), MPI, and lick efficiency. Subsequently, contrast tests were conducted comparing these same measures across the two amiloride conditions (photo-bleached and unadulterated; Tables 4 & 5). Preference score, burst duration, MPI, and lick efficiency for sodium solutions all vary significantly between the amiloride and control conditions, but not between amiloride conditions (Figures 8–11).

## Experiment 3: Preference for Light in Salt-Hungry Mice

**Purpose**

Our third aim was to study the role of Type I TRCs in salt taste by assessing the effect of selective stimulation of Type I TRCs in mice motivated to consume sodium. We hypothesized that mice genetically modified with light-activated Type I TRCS would prefer illuminated water over otherwise identical water when under conditions of sodium depletion.

**Subjects**

A total of 15 naïve male animals (nine ChR2; six Ai32) were used in this study ($37.91 \pm 1.56$ g).

**Procedure**

Approximately 48 hrs prior to testing, water bottles were changed on the home cage (water + 300 $\mu M$ amiloride). Access to food and water were not restricted. At the time of testing, animals were removed from the home cage and placed within a custom-built lickometer apparatus. Inside the chamber, animals had 30 min of free access to two bottles. Both bottles contained tap water, but one was modified with a 1 mm fiber optic cable producing 10 mW of light at a 470 nm (Figure 2), the standard wavelength for stimulating Channel Rhodopsin 2 (Nagel et al., 2003). Licking data on both spouts were collected by the apparatus, and recorded in Audacity before being exported to R for analysis. Each trial was repeated on the following day with the bottles reversed to account for any inherent side preference. Additionally, all animals completed an identical series of trials under sodium-replete (no amiloride) conditions.

**Results**

In order to assess the interaction of salt depletion and selective activation of Type I TRCs, we conducted a series of contrast tests measuring changes in licking behavior across genetic strain and depletion state. Our slate of contrasts first assessed effects of genetic strain by contrasting ChR2 and Ai32 subjects and then measured for effects of depletion state by contrasting depleted and non-depleted states for each strain individually (Tables 6 & 7). Effects of strain were pronounced: ChR2 mice demonstrated a significant preference for light relative to Ai32 controls ($F(1,49) = 27.67$, $p < 0.01$, $R^2 = 0.36$; Figure 12). This difference in preference was driven exclusively by the ChR2 animals' appetite for illuminated water,

which they licked significantly more often than Ai32 animals ($F(1,49) = 12.49$, $p < 0.01$, $R^2 = 0.20$), as there was no effect of strain on licking of water alone ($F(1,49) = 1.03$, $p = 0.32$, $R^2 = 0.02$; Figure 13). Similarly, ChR2 animals licked light in significantly larger bursts than Ai32 animals ($F(1,49) = 5.12$, $p = 0.03$, $R^2 = 0.09$; Figure 14), though lick efficiency was unaffected ($p = 0.23$).

The effects of depletion state add further insight: when salt depleted, ChR2 animals' preference for light increased significantly ($F(1,49) = 15.81$, $p < 0.01$, $R^2 = 0.24$). Again this difference in preference is driven by a change in behavior toward light rather than water: Licking of illuminated water increased significantly when the ChR2 animals were salt depleted ($F(1,49) = 35.66$, $p < 0.01$, $R^2 = 0.42$), but their licking of water alone was unchanged ($F(1,49) = 2.69$, $p = 0.11$, $R^2 = 0.05$). This result is in sharp contrast to the Ai32 mice, which did not change their behavior toward light when sodium-depleted ($F(1,49) = 0.28$, $p = 0.60$, $R^2 = 0.01$) and, in fact, slightly increased their licking of unilluminated water ($F(1,49) = 5.84$, $p = 0.02$, $R^2 = 0.11$; Figure 12). This increase in water drinking by the Ai32 controls is interesting, and may indicate the diuretic effect of amiloride, as Caloiero (2004) noted that animals in their experiment drank untreated water to recoup lost weight in a 24-hour break period between amiloride treatment and testing, an opportunity that our animals did not have.

**Experiment 4: Aversion for Light in Mice with a Conditioned Aversion to ENaC-Mediated or ENaC-Independent Salt Taste**

**Purpose**

Accepting that Type I TRCs contribute to salt taste, our next aim was to examine which neural pathway transduces that contribution. We hypothesized that if Type I TRCs transduce salt-taste information along an ENaC-Mediated pathway, a conditioned taste aversion to sodium gluconate, a compound thought to act exclusively on the ENaC-mediated salt-detection pathway (Breza & Contreras, 2012b; Geran & Spector, 2000; Ye, Heck, & DeSimone, 1993), should generalize to light stimulation of Type I TRCs.

**Subjects**

Sixteen ChR2 mice were be used, including eight male and eight female mice.

**Procedure**

Animals received two training sessions with the apparatus. Water was removed from the home cage 24 hrs prior to testing. On the day of testing, mice were removed from the home cage and placed in the testing chamber, where they had 30 min of free access to two bottles, one containing 0.2 $M$ sucrose and the other containing tap water. The procedure was repeated with the bottles reversed on the following day, after which water was replaced on the home cage.

After a break of two days, taste aversion conditioning began. Water was removed from the home cage 24 hrs prior to acquisition trials. On Days 1 and 3, animals were weighed and transferred from the home cage to the testing chamber, where they were given 15 min of ad-libitum exposure to 0.1 $M$ sodium gluconate. Immediately after the trial, experimental animals were given an injection of 0.15 $M$ lithium chloride (2.0 mEq/kg body wt). Control animals received an equivalent injection of 0.15 $M$ NaCl. Five hours after the trial, they were given 15 minutes of free access to tap water in the home cage. On Day 2, animals were again weighed and placed in the testing chamber, where they were given 15 min of free access to one bottle containing tap water before being returned to the home cage. Five hours after testing, they received 15 min of free access to tap water in the home cage. On Days 4 and 5, animals were

removed from the home cage and given 15 min of free access to two bottles, both of which contained tap water. Additionally, one bottle was threaded with a 1mm optic fiber producing 10 mW of light of a wavelength of 470 nm. Bottle position was reversed between Days 4 and 5.

**Data Analysis**

Lickometer recordings produced in Audacity were exported to R and analyzed by a lick-counting program to assess preference between water and water with light, compared across control and experimental conditions.

**Results**

There was no significant difference in preference for light between the control and experimental groups ($p = 0.47$; Figure 15). MPI for illuminated water was significantly shorter ($p = 0.04$, $d = 0.73$), and lick efficiency was significantly lower ($p = 0.05$, $d = 0.73$), but licks per burst did not significantly vary ($p = 0.20$), nor did the total count of licks to light ($p = 0.10$) or water ($p = 0.94$; Figure 16). Overall, it appeared that lithium injections did not substantially or reliably alter behavior toward light (Table 8). As such, we decided to verify the aversion, and found that the animals given lithium injections demonstrated no change in lick total between the first and second acquisition trials ($p = 0.86$). Similarly, there was no change in licks per burst ($p = 0.93$), lick efficiency ($p = 0.43$), or MPI ($p = 0.76$; Figure 17). Descriptive statistics are given in Table 9. As such, it appears that mice did not form an aversion to the conditioned stimulus, sodium gluconate.

**Discussion**

Salt taste plays a crucial role in our interactions with food, which in turn means that salt has a considerable impact on our lives. Historically, this has hinged on the role of sodium as a scarce and essential nutrient, shaping our society in ways that we scarcely comprehend (Kurlansky, 2002). More recently, that scarcity has reversed itself, and we now routinely contend with the detrimental health effects of excessive sodium consumption (Ma et al., 2015; Polonia et al., 2016). This makes the study of salt consumption an important issue in public health, of which salt taste is an obviously relevant component.

Despite this, the mechanisms of salt taste remain poorly understood. It is understand that most, but not all, of the gustatory nerve responses to salt rely on epithelial sodium channels (Heck et al., 1984), but it is not know what type of taste-bud cell transduces salt taste via this mechanism. The receptor of ENaC-independent salt-taste has not identified, let alone the variety of cells that house them. It has been established that there are neuronal subtypes in various taste-processing centers of the nervous system that respond preferentially to sodium (Breza et al., 2010; Frank, 1974; Nishijo & Norgren, 1990). It is unknown, however, what receptor cells they communicate with. With this series of experiments, our goal was to begin to shed some light on these cellular mechanisms.

Our first task was the development of an affordable mechanism to measure taste-guided behavior in mice. Once we discovered that a circuit routed through a standard desktop microphone input could act as a serviceable electronic lickometer, we were able to refine that mechanism into a behavioral chamber and write a program to identify licks in the resulting electrical recording. Having done that, we were able to successfully validate our program against professional spike-counting software and confirm that both programs recorded patterns of licks that reflected those observed by previous researchers. While our mechanism is limited to free-access trails of a small number of solutions (up to four), our design offers one feature missing from most other lickometers published or sold: accessibility. A combination of free software and simple materials (Raymond et al., 2018) makes our design ideal for a number of uses, particularly teaching, or in our case, basic preference testing.

Having developed a tool to measure behaviors tied to salt taste, our next objective was to guarantee that we could reliably manipulate those behaviors. As our ultimate goal was to optogentically activate Type I TRCS to simulate salt taste, we required a clear behavioral indicator of salt taste to serve as a comparison. Salt hunger stood out as an obvious candidate, and our next experiment explored a method for inducing sodium appetite in mice. Using a previous study in rats as a template (Caloiero & Lundy, 2004), we were able to use self-administered amiloride to rapidly drive consumption of salt. This technique provided several benefits relative to dietary sodium restriction: Amiloride was able to induce salt appetite more quickly and affordably than altering the animals' diet. More importantly, mice could consume high concentrations of sodium in the testing chamber and still demonstrate sodium appetite on the next day, a crucial feature allowing us to repeat two-bottle preference-tests on back-to-back days, reversing bottles to account for side preference. A secondary dimension of this experiment also produced interesting results; our attempt to create an inactive control for amiloride (which is known to be light-sensitive) by destroying it with prolonged direct exposure to a powerful incandescent light proved totally ineffective, suggesting that the drug is fairly resistant to artificial light. This is not totally surprising, as previous studies have shown amiloride to be effective without taking special precautions to protect it against (presumably less intense) light exposure (Caloiero & Lundy, 2004). It is worth noting that epithelial sodium channels are apical on TRCs (Breza & Contreras, 2012b), meaning that any amiloride lingering in the oral cavity of the subjects subsequent to self-administration is immediately washed away from the TRCs when the mice lick the taste solutions in the testing chamber. This is important, as it prevents the amiloride self-administered in the home cage from having a direct, suppressive impact on sodium detection in the testing chamber, confirmed by the sodium appetite demonstrated in these trials (topical amiloride in taste solutions inhibits sodium appetite; Bernstein & Hennessy, 1987).

With the second experiment completed, we had everything we needed to begin our examination of the Type I TRCs: an apparatus capable of measuring licks, an amiloride treatment capable of inducing licking of sodium solutions, and a transgenic animal with light-activated Type I TRCs. We hypothesized that if the Type I TRCs play a role in transducing the taste of sodium, treating the transgenic animals with

amiloride should motivate them to lick illuminated water as though it contained sodium. Using a light-insensitive transgenic parent strain as a control group in addition to sodium replete animals of both strains allowed us to directly assess simulated sodium appetite, controlling for the potential of incidental preference for light. The results were conclusive; sodium-depleted light-sensitive animals consumed light voraciously, and animals in any other condition did not. Stimulation of the Type I TRCs simulates a taste that sodium-depleted animals are driven to consume. This alone does not clarify the exact taste being simulated, but it does guarantee that there is a sodium-like component in the taste being produced. Further, it seems that light is simulating ENaC-mediated sodium taste (rather than the ENaC-independent pathway alone) as previous research has demonstrated that sodium depletion does not drive consumption of non-sodium salts (Roitman & Bernstein, 1999), nor even sodium salts when ENaC has been suppressed by topical amiloride (Bernstein & Hennessy, 1987).

Experiment 3 demonstrated that selective activation of the Type I TRCs simulates ENaC-mediated salt taste, but it did not address the question of whether additional tastes were being simulated simultaneously. In an attempt to examine that issue more closely, we next attempted to condition an aversion selectively to the ENaC-mediated salt taste pathway by pairing injections of lithium chloride with exposure to sodium gluconate, which preferentially activates the amiloride-sensitive component of salt taste (Breza & Contreras, 2012b; Smith, Treesukosol, Paedae, Contreras, & Spector, 2012).  It was our hypothesis that if an aversion to sodium gluconate generalized to light, we could conclude that light stimulation of Type I TRCs also preferentially simulated ENaC-mediated taste. Unfortunately, results were inconclusive. While some of the microstructural indicators of palatability were significantly shifted in experimental mice relative to controls, others were not, nor were more obvious indications of appetite like total lick count or even preference for light relative to water. Confused by these results, we went back and examined the data from the acquisition trials to confirm whether we had successfully conditioned an aversion and found similarly dubious results. Comparing the first and second acquisition trials of the lithium-injected mice yielded no significant differences, suggesting that the aversion to sodium gluconate was not effectively conditioned. This leaves open the question of what exactly the light tastes like when it

stimulates the Type I TRCs: Experiment 3 established an ENaC-mediated component, but the possibility of additional side-band tastes remains.

**Conclusion**

      In the series of experiments described above, we were able to validate a simple lickometer, confirm a method for rapid induction of sodium appetite in mice, and use optogenetic techniques to simulate salt taste by stimulating Type I TRCs directly. These findings are significant for several reasons. Primarily, they answer the core question motivating the experiments; Type I TRCs make some substantial contribution to the detection of sodium by the taste system. This information contains a dual importance, as it satisfies two previously unanswered questions simultaneously by identifying a TRC type that detects salt and identifying a taste stimulus detected by Type I TRCs. The secondary importance of these experiments follows from the significance of that finding; they are a further proof of concept in the ongoing movement toward open science. All of the experiments detailed here were conducted under a mandate of affordability, a guideline explicit in the first two experiments and implicitly interwoven in the remainder as a result. Not only were we able to demonstrate that Type I TRCs transduce sodium taste, we were able to demonstrate that cutting-edge research in the field of taste can be accomplished affordably.

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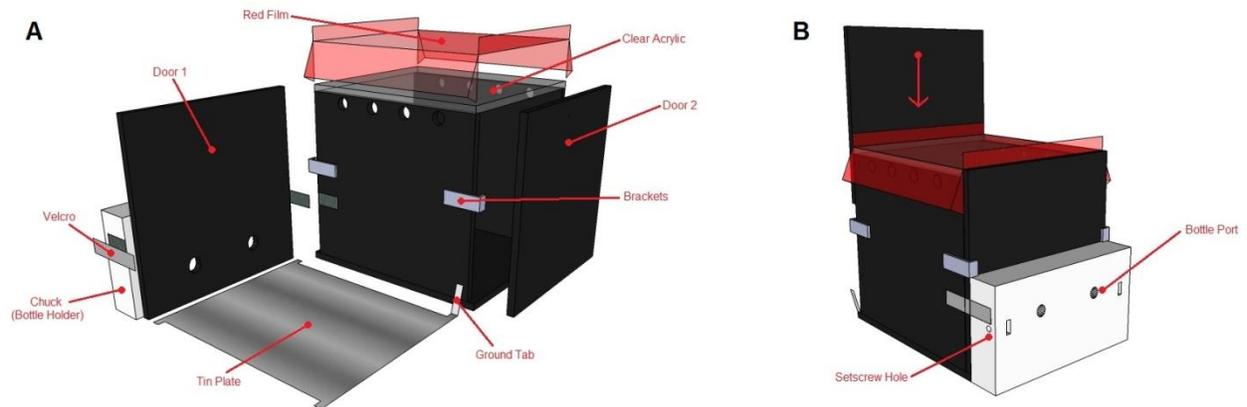
**Figures**



*Figure 1.* Computer rendering of the lickometer apparatus. A: Illustration of the lickometer chamber, exploded. The main body of the chamber is composed of cut acrylic sheets, with acrylic doors that slide into place on bracketed tracks. A delrin bottle holder attached to the chamber via velcro, and a tin plate cut and fitted to the chamber interior serves as an electrical ground. B: The lickometer chamber, assembled and rotated 180°.
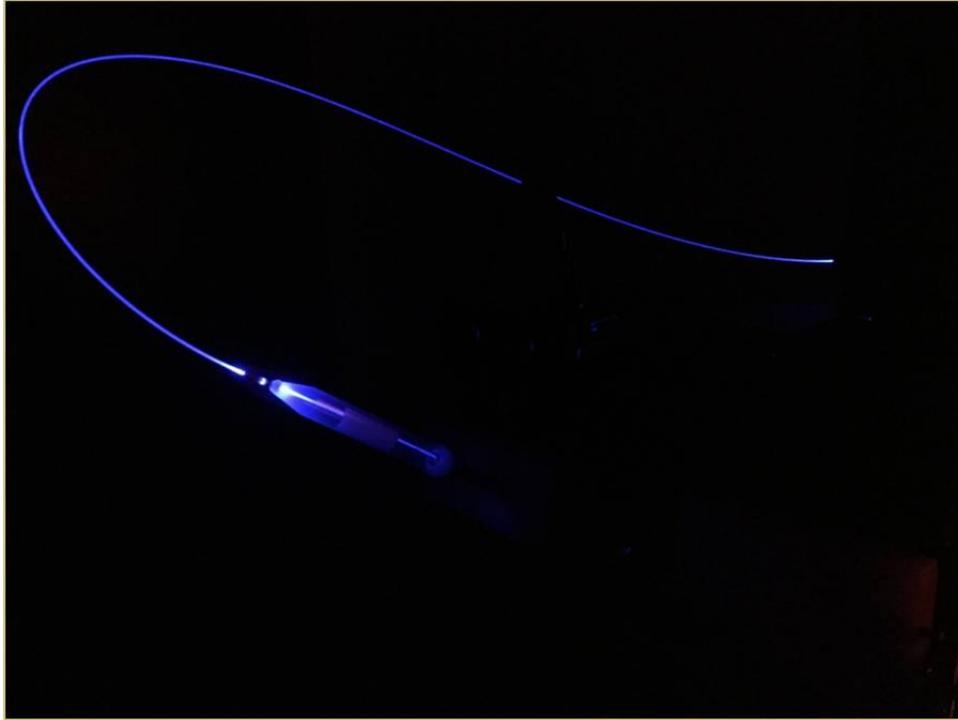
*Figure 2*. Photograph of an optic fiber cable passed through a water bottle. The fiber terminates just inside the aperture of the bottle's spout. Thus positioned, the fiber illuminates the subject's tongue whenever the spout is licked.
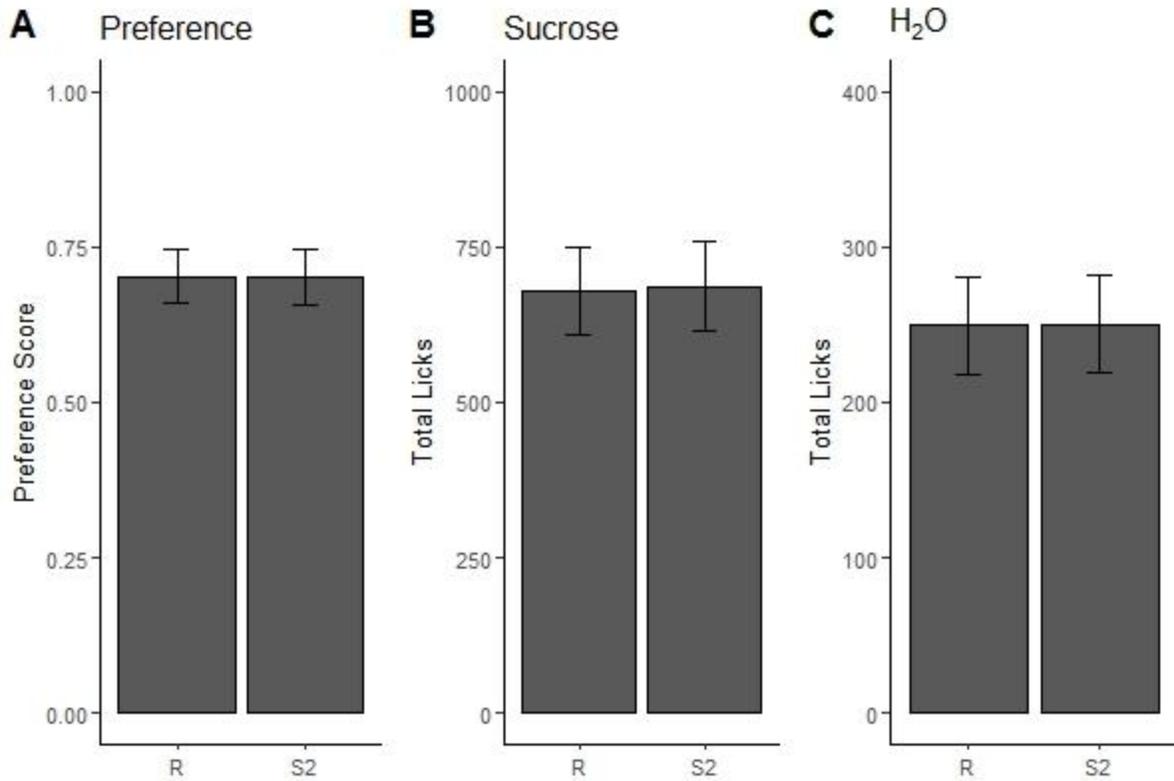
*Figure 3.* Graphs illustrating the licks counted by two software packages; R and Spike 2 (S2). Shown are records of calculated preference scores (A), and mean licks recorded to sucrose (B) and water (C). By all three measures, the counts produced by both programs are virtually identical.
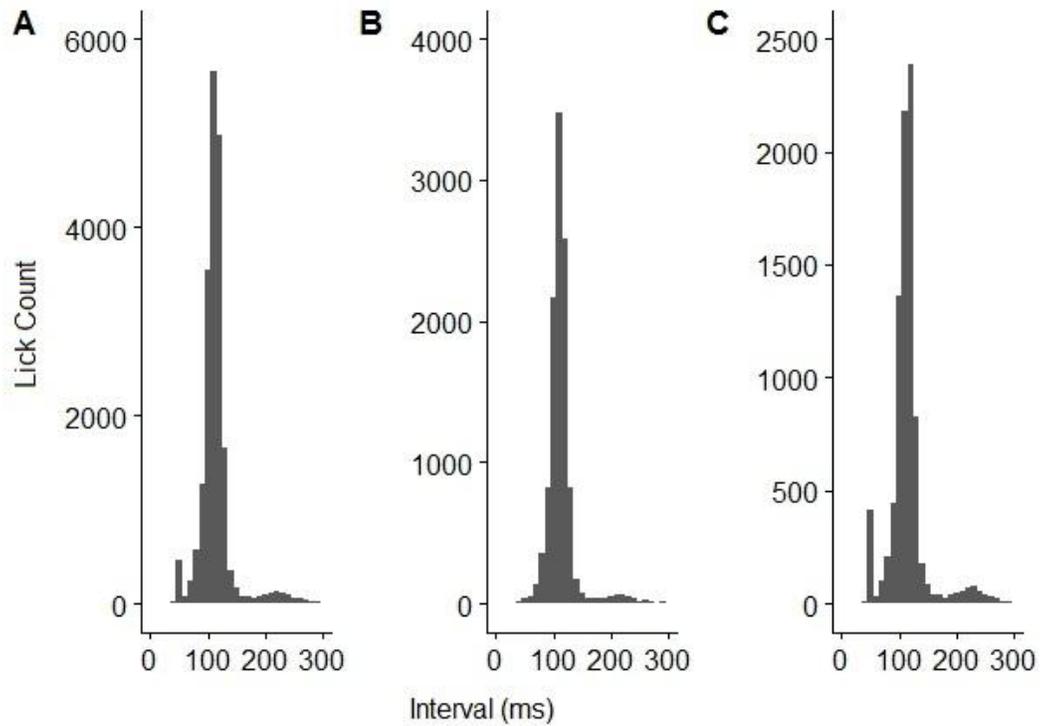
*Figure 4.* Histogram of interlick intervals recorded in Experiment 1. Histograms show all ILIs recorded across all test sessions (A), or separated by those with no resistance added (B), or using 10 MΩ of additional resistance (C). The distribution of ILIs peaks at 110 ms, with a secondary distribution around 220 ms, a phenomenon documented in previous lickometer research (Glatt et al., 2016).
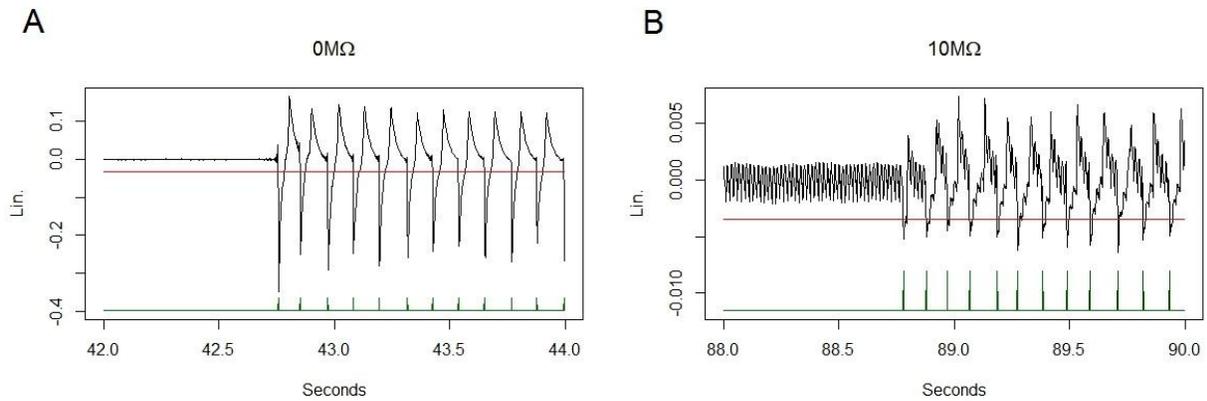
*Figure 5.* Traces of the raw lickometer signal, each showing a recording of a two-second period of time. The red line indicated the threshold for detection of a lick, and the green line indicates when the lick-counting program has registered a lick and recorded the event. One trace was recorded on a circuit with no added resistance (A), the other on a circuit with 10 MΩ of added resistance. It is evident that adding resistance to the recording circuit dramatically decreases the magnitude of the lickometer signal, severely reducing the signal-to-noise ratio.
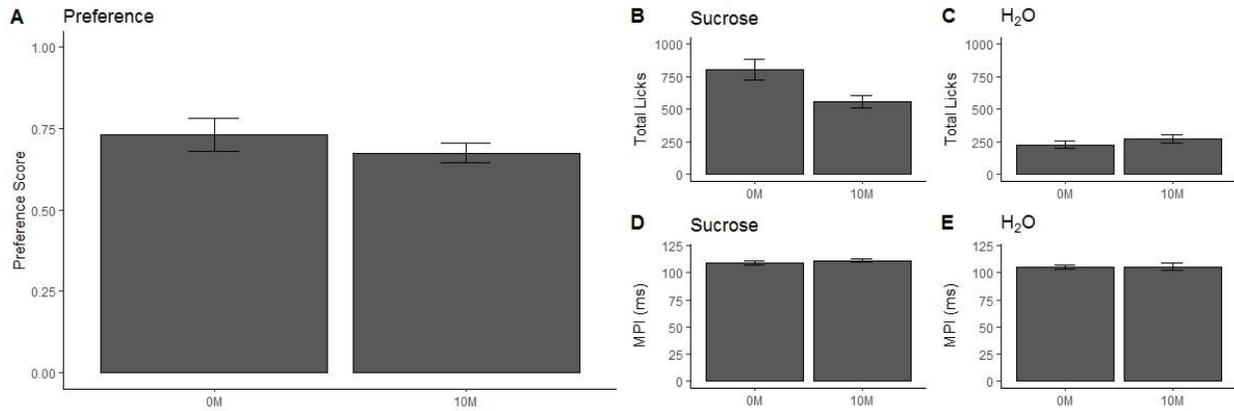
*Figure 6.* Graphs of licking data separated by resistance added to the recording circuit. Only one difference shown is significant; mice licked sucrose (B) significantly more often when no resistance was added to the circuit ($p = 0.03$, $d = 1.53$). Given the lack of other significant differences, we are inclined to interpret this as a practice effect, as the same mice had previously undergone the same testing procedure with 10 M$\Omega$ of resistance added to the recording circuit.
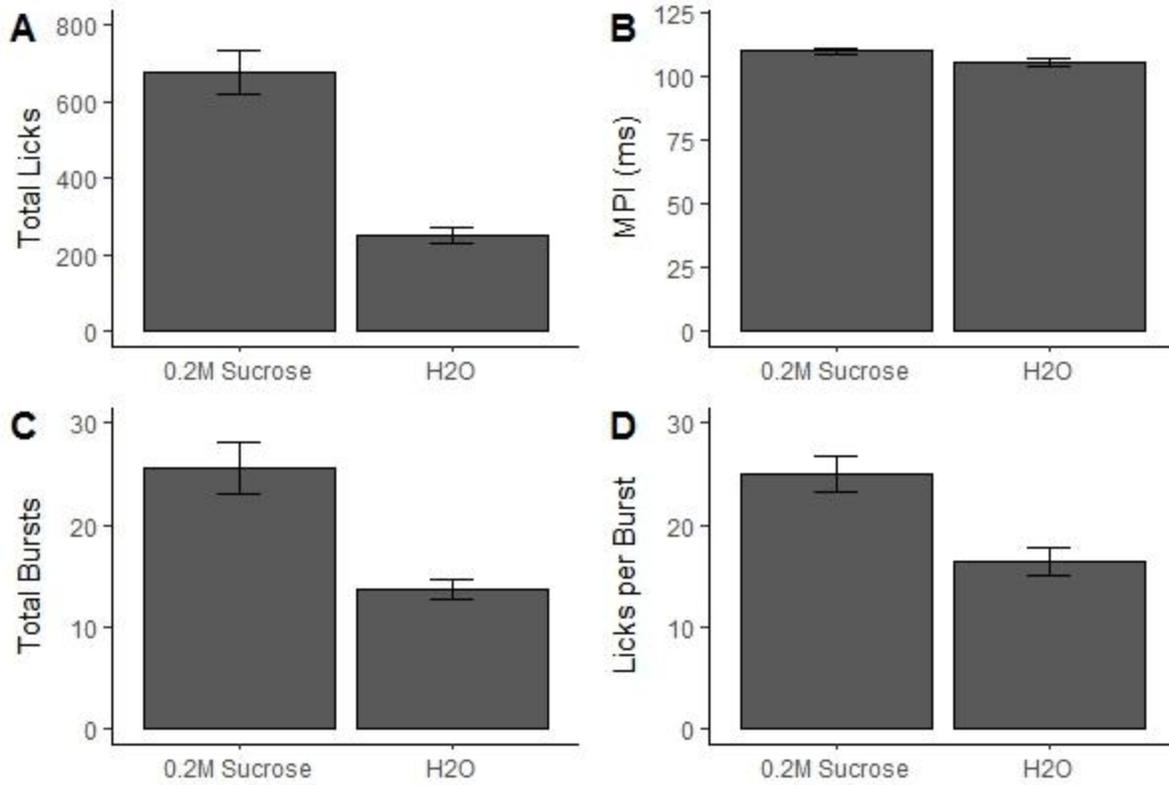
*Figure 7.* Graphs of palatability indicators as a function of taste solution. Findings show that mice in our lickometer apparatus tended to find 0.2 *M* sucrose more palatable than tap water. All differences are significant ($p < 0.05$).
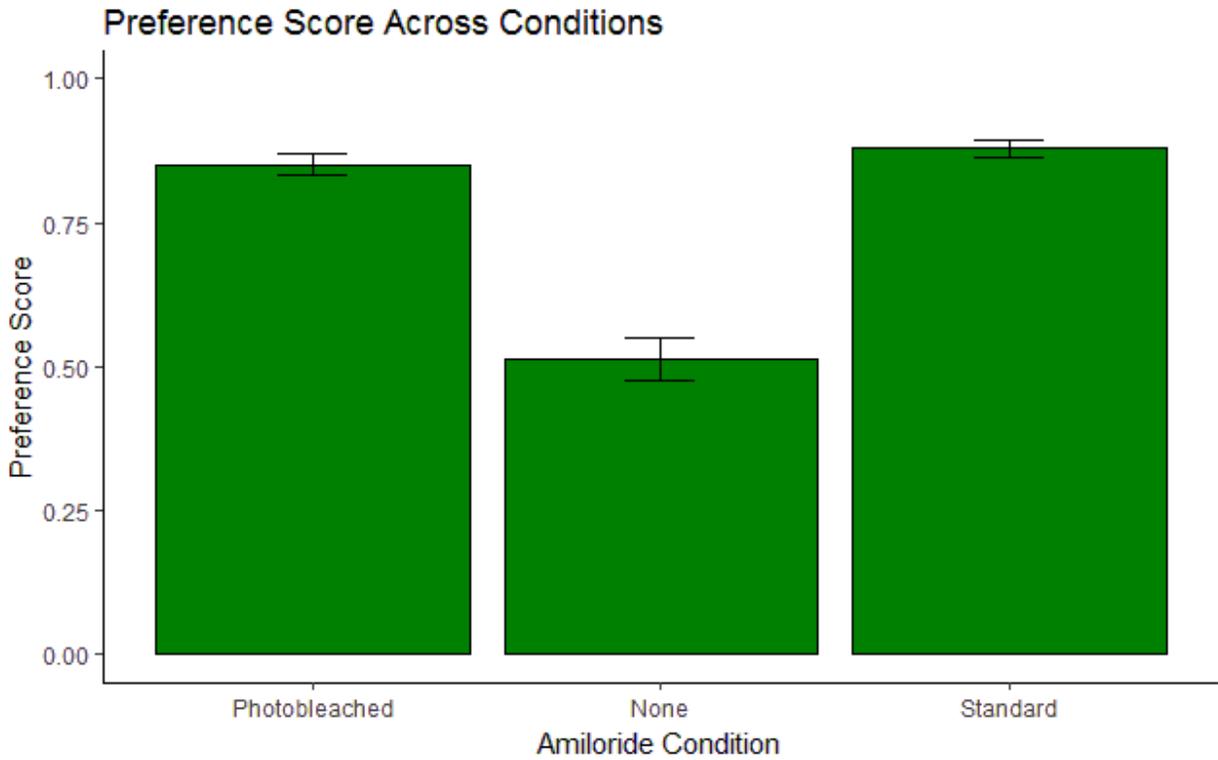
*Figure 8.* Preference for 0.3 *M* NaCl solution across different amiloride treatment conditions. There was

no difference in preference between mice treated with unadulterated or light-treated amiloride ($p = 0.45$),

but those two groups had a significantly higher preference for NaCl than the untreated control condition

($p < 0.01$), indicating that self-administration of 300 $\mu M$ amiloride is sufficient to drive sodium appetite in
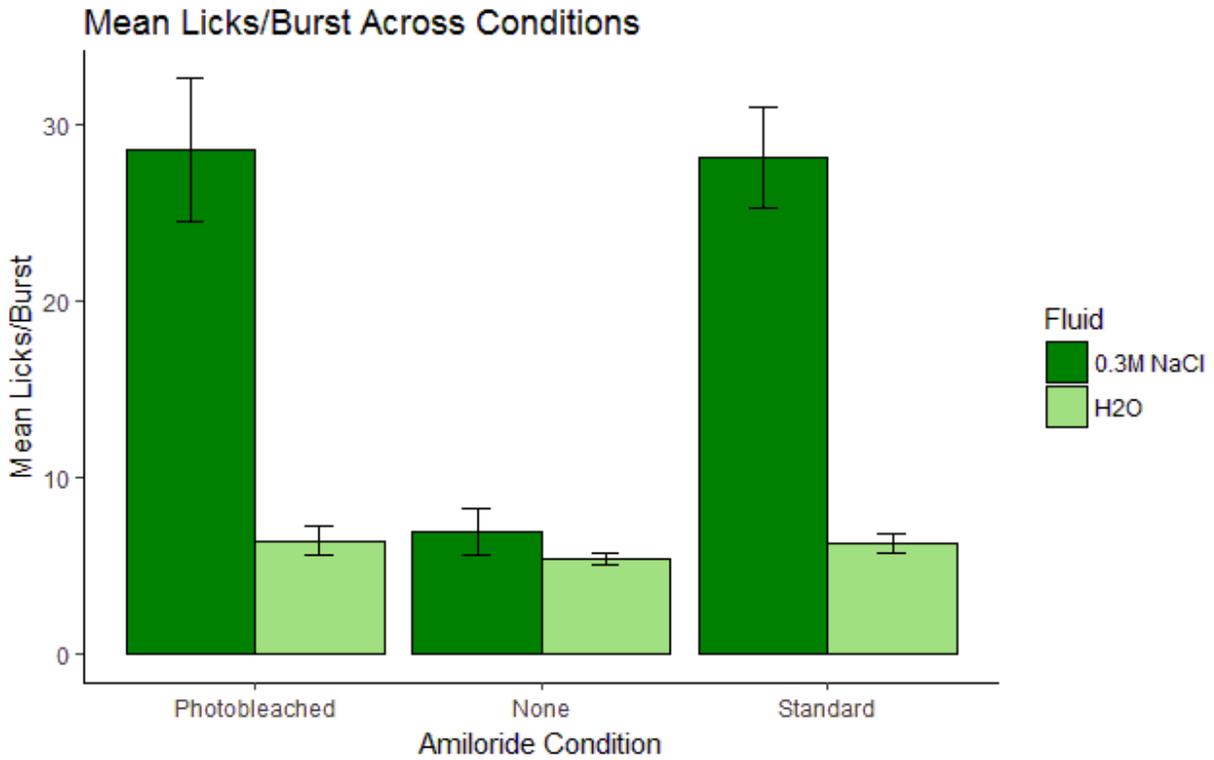
mice.

*Figure 9.* Mean licks per burst separated by amiloride treatment condition. Mice treated with amiloride drank NaCl solution in significantly longer bursts than untreated control animals ($p < 0.01$), with no difference between treatment groups ($p = 0.92$).
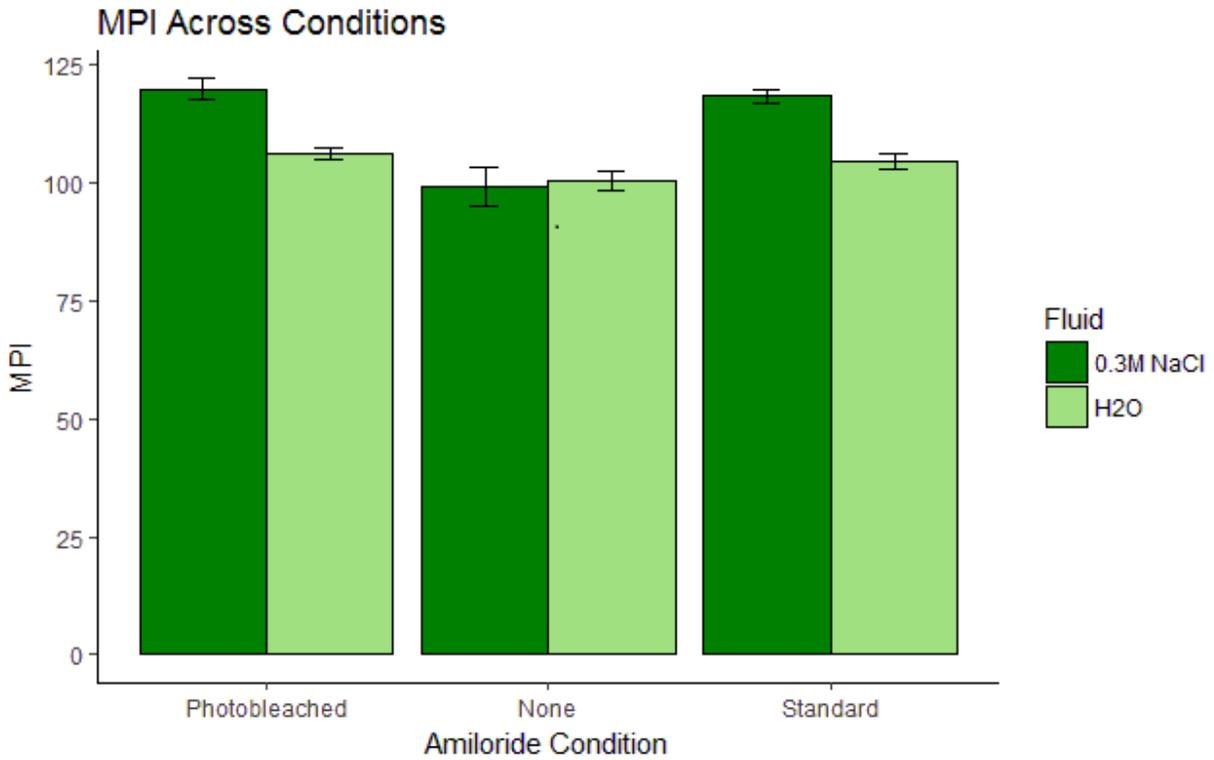
*Figure 10.* MPI separated by amiloride treatment condition. Mice treated with amiloride had significantly longer MPIs than untreated control animals ($p < 0.01$), but there was no difference between treatment groups ($p = 0.10$). It has been proposed that longer MPIs may indicate increased palatability (Glatt et al., 2016).
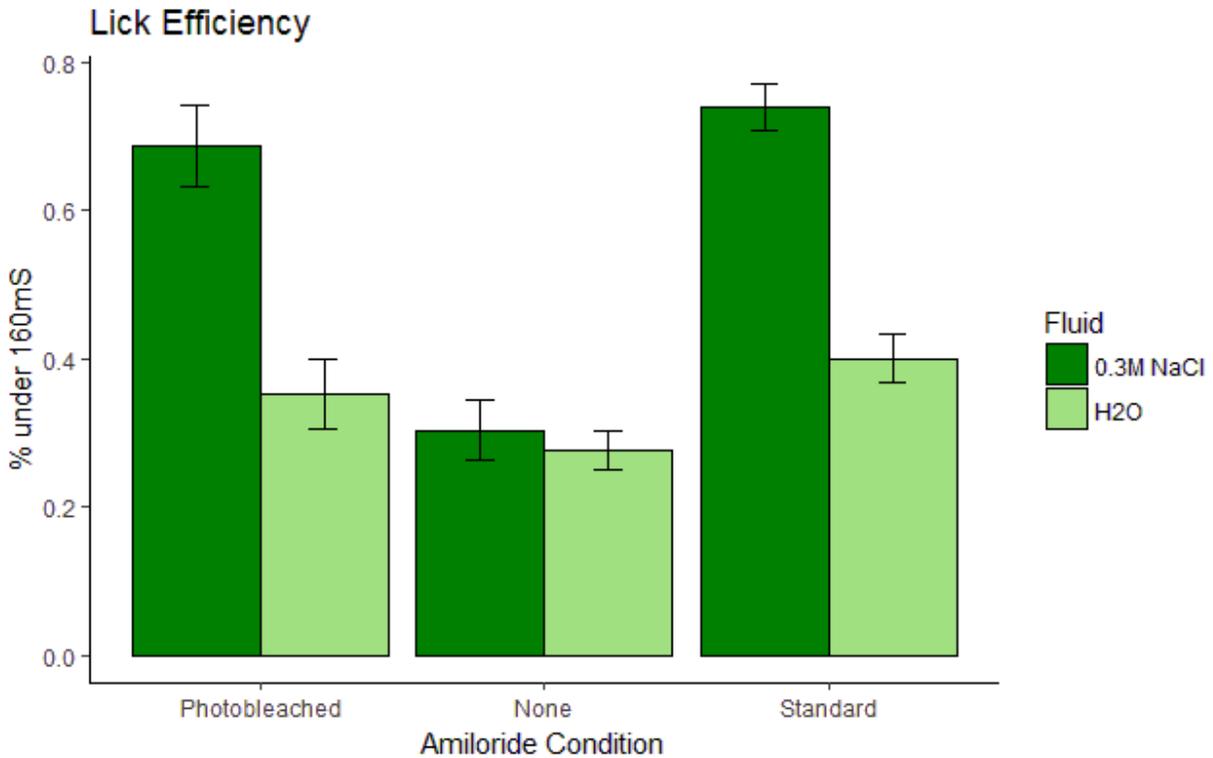
*Figure 11.* Lick efficiency across amiloride treatment condition. The licks to NaCl solution of mice treated with amiloride occurred mainly in bursts, unlike untreated control mice. The difference is significant ($p < 0.01$). This indicates that when treated with amiloride, mice were more likely to taste NaCl solution and continue drinking, rather than sampling and retreating.
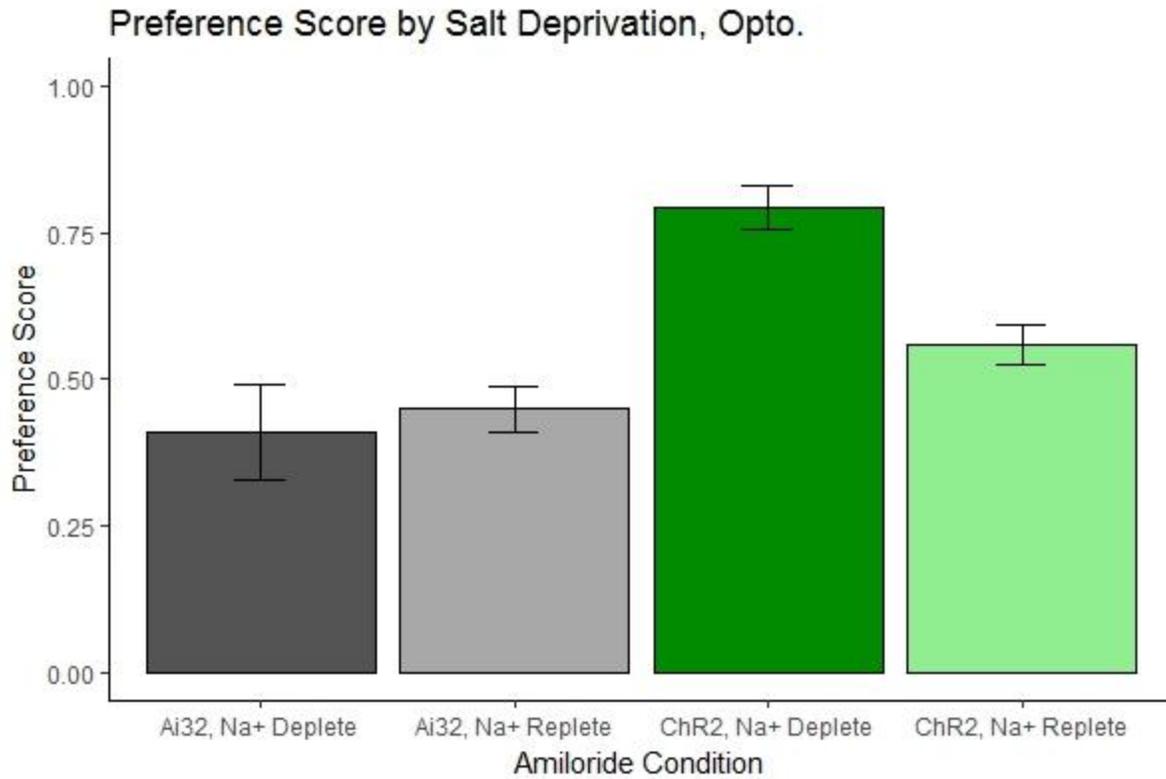
*Figure 12.* Preference for illuminated water over plain tap water. Mice with light-sensitive Type I TRCs had a significantly higher preference for light than light-insensitive mice ($p < 0.01$), a preference that dramatically increased when the light-sensitive mice were sodium-depleted ($p < 0.01$). Sodium depletion had no effect on light-insensitive mice ($p = 0.60$). These findings indicate that stimulation of the Type I TRCs simulates the taste of sodium.
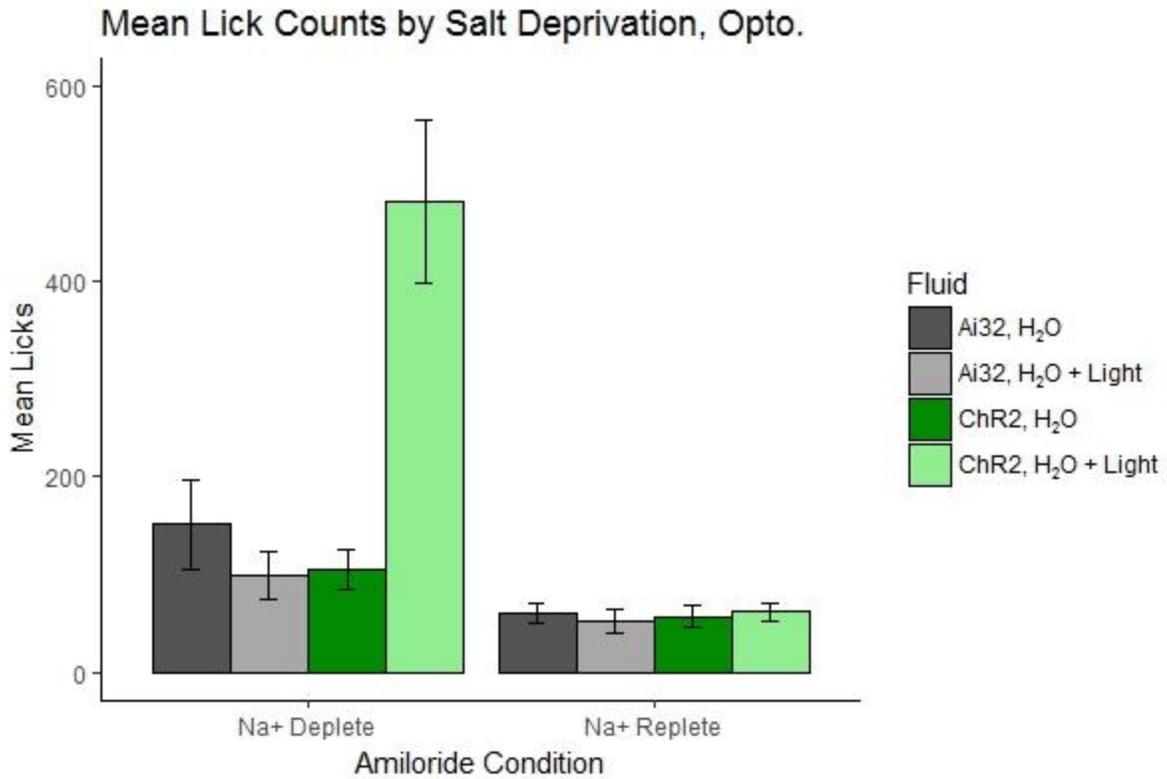
*Figure 13.* Mean licks recorded to water or illuminated water across condition of sodium depletion. There was a significant effect of sodium depletion on licks to light in the light-sensitive animals ($p < 0.01$), but not the light-insensitive animals ($p = 0.60$). Additionally, sodium-replete animals demonstrated no difference in licking of light across genetic strain ($p = 0.53$).
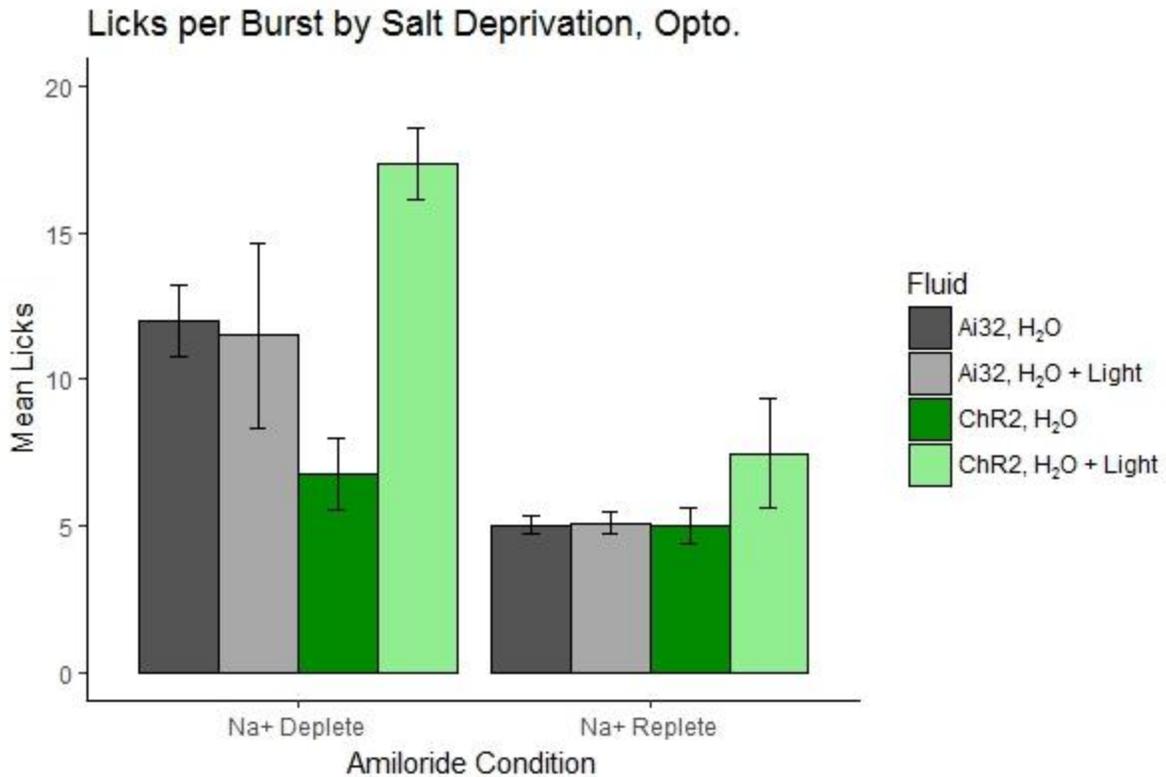
*Figure 14.* Average number of licks per burst of licks across amiloride treatment condition. Genetic strain had a significant effect on burst duration, with light-sensitive animals licking in longer bursts than controls ($p = 0.03$). Sodium-depletion had a significant effect on the duration of bursts delivered to light for both strains ($p < 0.05$), but in control mice, this increase was not significantly different than their increased burst duration for water ($p = 0.89$). Light-sensitive mice, on the other hand, had significantly longer bursts to light compared to water when sodium-depleted ($p < 0.01$).
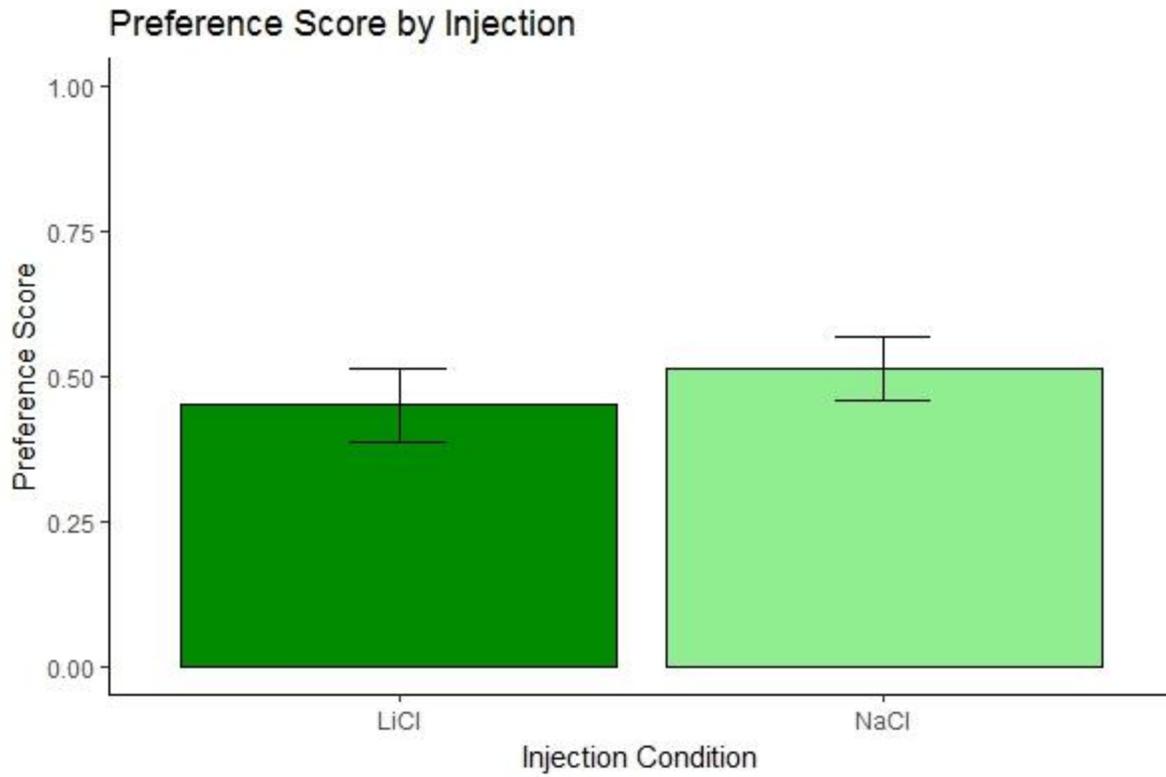
*Figure 15.* Preference for light relative to water in light-sensitive mice compared across CTA conditions.

Whether mice were injected with NaCl or LiCl during pairings with sodium gluconate had no significant

effect on preference score ($p = 0.47$).
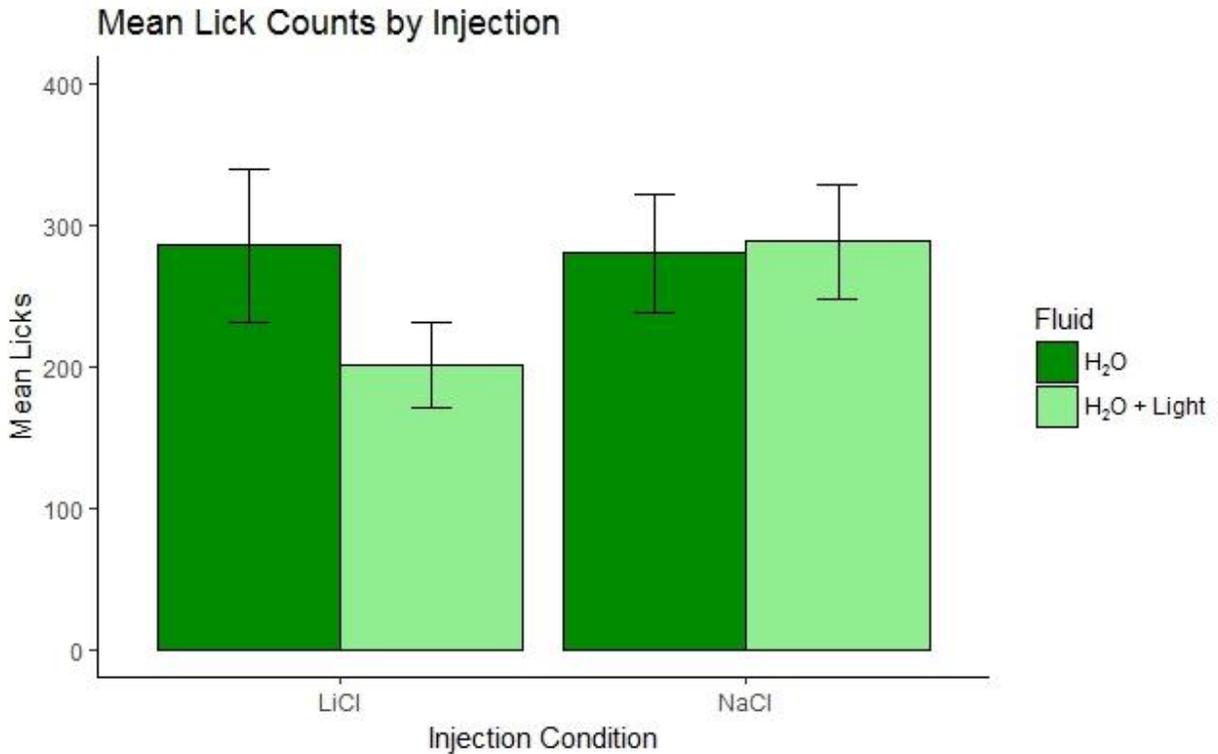
*Figure 16.* Mean licks counts to light and water compared across CTA condition. There were no significant differences in total lick count, regardless of whether mice were injected with LiCl or NaCl during acquisition trials. Combined with the insignificant difference in preference score, these results indicate that mice who experienced the taste of sodium gluconate paired with lithium injections did not significantly avoid light.
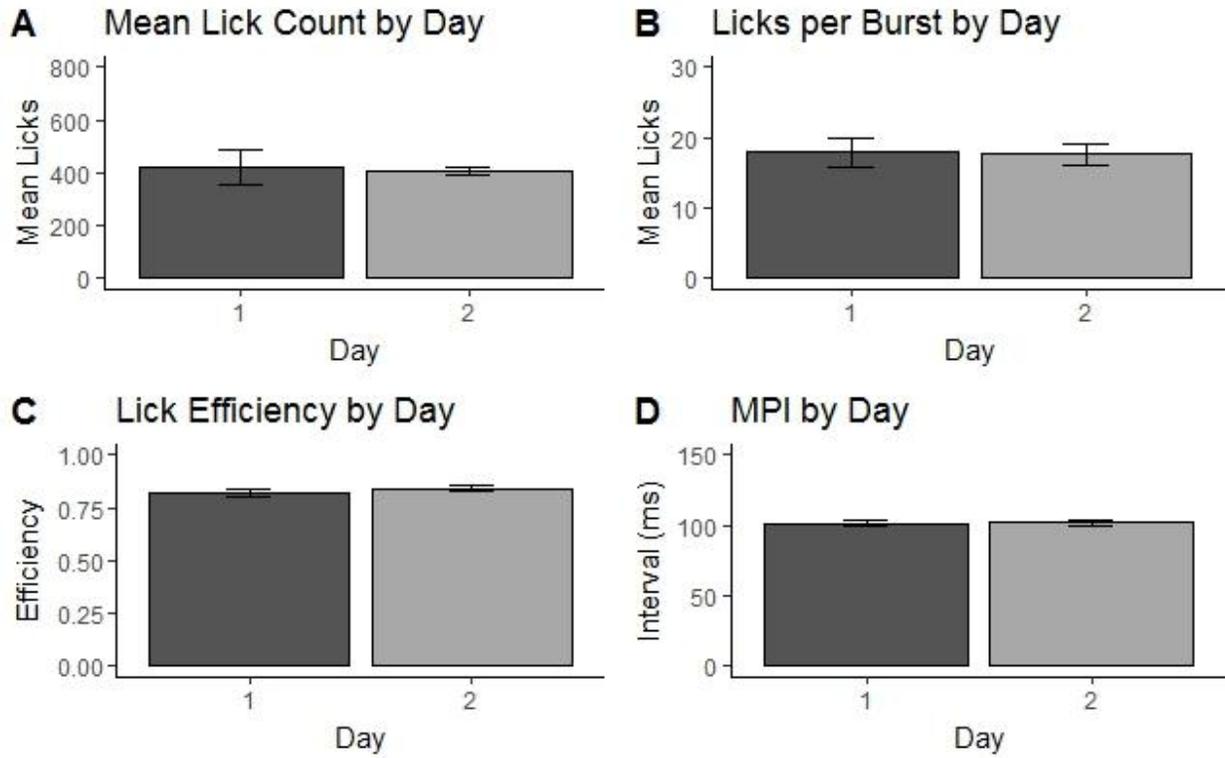
*Figure 17.* Various microstructural indicators of palatability measured during acquisition trails with sodium gluconate. There were no significant differences, indicating that injections of 0.15 *M* lithium chloride failed to condition an aversion to 0.1 *M* sodium gluconate.

**Tables**

Table 1

*Comparisons of Licks Identified by Two Different Software Packages Processing the Same Set of Lickometer Recordings*

|  | R | | Spike2 | | | | |
|---|---|---|---|---|---|---|---|
|  | Mean | SE | Mean | SE | *p* | *d* | *r* |
| Water Licks | 249.62 | 31.59 | 250.38 | 31.61 | 0.99 | 0.00 | 1.00 |
| Sucrose Licks | 679.62 | 70.05 | 685.88 | 71.76 | 0.95 | 0.02 | 1.00 |
| Preference Score | 0.70 | 0.04 | 0.70 | 0.04 | 0.99 | 0.01 | 1.00 |

*Notes.* SE indicates standard error, and *d* indicates Cohen's *d*.

Table 2

*Measurements of Several Indicators of Palatability, Compared Across Sessions with Either no Added Resistance or 10 MΩ of Resistance Added to the Recording Circuit*

| | 0 MΩ | | 10 MΩ | | | |
|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | *p* | *d* |
| Preference Score | 0.73 | 0.05 | 0.67 | 0.03 | 0.38 | 0.53 |
| Sucrose Licks | 801.83 | 77.84 | 557.42 | 49.38 | 0.03 | 1.53 |
| Water Licks | 226.75 | 29.66 | 272.50 | 32.29 | 0.32 | 0.60 |
| Sucrose MPI | 108.70 | 2.19 | 111.17 | 1.64 | 0.39 | 0.52 |
| Water MPI | 105.34 | 1.87 | 105.42 | 3.14 | 0.98 | 0.01 |

Table 3

*Comparisons of Consumption of 0.2 M Sucrose and Tap Water During Experiment 1*

|  | Sucrose | | Tap Water | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Mean | SE | Mean | SE | p | *d* | *r* |
| Total Licks | 679.62 | 57.35 | 249.62 | 22.01 | 0.00 | 2.86 | -0.25 |
| MPI | 109.93 | 1.36 | 105.38 | 1.74 | 0.05 | 0.84 | 0.61 |
| # of Bursts | 25.54 | 2.53 | 13.71 | 1.01 | 0.00 | 1.77 | 0.22 |
| Licks per Burst | 25.04 | 1.79 | 16.46 | 1.38 | 0.00 | 1.55 | 0.80 |

Table 4

*Contrast Tests Comparing Several Measures of Palatability Across Animals Treated with Amiloride or Water and Across Animals Treated with Photo-Bleached Amiloride or Unadulterated Amiloride*

| | Amiloride vs $H_2O$ | | | Photo-Bleached Amiloride vs Standard Amiloride | | |
|---|---|---|---|---|---|---|
| | $F(1,33)$ | $p$ | $R^2$ | $F(1,33)$ | $p$ | $R^2$ |
| Preference Score | 127.90 | < 0.01 | 0.79 | 0.58 | 0.45 | 0.02 |
| Total Licks (NaCl) | 28.18 | < 0.01 | 0.46 | 0.57 | 0.46 | 0.02 |
| Total Licks ($H_2O$) | 2.33 | 0.14 | 0.07 | 0.32 | 0.57 | 0.01 |
| Licks/Burst (NaCl) | 34.93 | < 0.01 | 0.51 | 0.01 | 0.92 | 0.00 |
| Lick Efficiency (NaCl) | 59.51 | < 0.01 | 0.64 | 0.70 | 0.41 | 0.02 |

Table 5

*Descriptive Statistics for a Number of Indicators of Palatability, Separated by Amiloride Treatment Condition*

|  | Amiloride | | Photo-Bleached Amiloride | | Water | |
|---|---|---|---|---|---|---|
|  | Mean | SE | Mean | SE | Mean | SE |
| Preference Score | 0.88 | 0.01 | 0.85 | 0.02 | 0.51 | 0.04 |
| Total Licks (NaCl) | 782.25 | 96.39 | 678.25 | 138.45 | 94.17 | 15.93 |
| Total Licks ($H_2O$) | 116.67 | 14.91 | 106.50 | 13.71 | 87.92 | 8.39 |
| Licks/Burst (NaCl) | 28.11 | 2.85 | 28.55 | 4.04 | 6.96 | 1.32 |
| Lick Efficiency (NaCl) | 0.74 | 0.03 | 0.69 | 0.05 | 0.30 | 0.04 |

Table 6

*Contrast Tests Assessing Effects of Genetic Strain, and of Sodium Depletion Within Strains, on a Number of Indicators of Palatability of Water or Water Illuminated with 470 nm Light*

|  | ChR2 vs. Ai32 | | | Dep vs. Rep (ChR2) | | | Dep vs. Rep (Ai32) | | |
|---|---|---|---|---|---|---|---|---|---|
|  | $F(1,49)$ | $p$ | $R^2$ | $F(1,49)$ | $p$ | $R^2$ | $F(1,49)$ | $p$ | $R^2$ |
| Preference Score | 27.67 | $< 0.01$ | 0.36 | 15.81 | $< 0.01$ | 0.24 | 0.29 | 0.60 | 0.01 |
| Total Licks (470 nm) | 12.49 | $< 0.01$ | 0.20 | 35.66 | $< 0.01$ | 0.42 | 0.28 | 0.60 | 0.01 |
| Total Licks ($H_2O$) | 1.03 | 0.32 | 0.02 | 2.69 | 0.11 | 0.05 | 5.84 | 0.02 | 0.11 |
| Licks/Burst (470 nm) | 5.12 | 0.03 | 0.09 | 18.26 | $< 0.01$ | 0.27 | 4.66 | 0.04 | 0.09 |
| Efficiency (470 nm) | 1.47 | 0.23 | 0.03 | 49.22 | $< 0.01$ | 0.50 | 14.56 | $< 0.01$ | 0.23 |

Table 7

*Descriptive Statistics for a Number of Indicators of Palatability of Water or Water Illuminated with 470 nm Light, Separated by Genetic Strain and Amiloride Treatment Condition*

| | ChR2-Dep | | ChR2-Rep | | Ai32-Dep | | Ai32-Rep | |
|---|---|---|---|---|---|---|---|---|
| | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Preference Score | 0.79 | 0.04 | 0.56 | 0.03 | 0.41 | 0.08 | 0.45 | 0.04 |
| Total Licks (470 nm) | 482.06 | 83.83 | 61.62 | 9.88 | 99.80 | 24.71 | 51.80 | 11.63 |
| Total Licks ($H_2O$) | 105.24 | 21.15 | 56.81 | 11.75 | 152.00 | 45.88 | 60.30 | 9.66 |
| Licks/Burst (470 nm) | 17.37 | 1.24 | 7.46 | 1.88 | 11.51 | 3.16 | 5.08 | 0.37 |
| Efficiency (470 nm) | 0.65 | 0.02 | 0.31 | 0.03 | 0.55 | 0.07 | 0.32 | 0.02 |

Table 8

*Descriptive Statistics for a Number of Indicators of Palatability, Separated by CTA Condition. Data Collected during Generalization Trials Comparing Water and Water Illuminated with 470 nm Light*

| | LiCl Injection | | NaCl Injection | |
|---|---|---|---|---|
| | Mean | SE | Mean | SE |
| Preference Score | 0.45 | 0.06 | 0.51 | 0.06 |
| Total Licks (470 nm) | 201.38 | 30.00 | 288.56 | 40.63 |
| Total Licks (H$_2$O) | 285.75 | 54.56 | 280.38 | 41.80 |
| Licks/Burst (470 nm) | 20.71 | 2.99 | 27.22 | 3.93 |
| Efficiency (470 nm) | 0.75 | 0.05 | 0.85 | 0.02 |
| MPI (470 nm) | 95.72 | 4.08 | 107.43 | 3.69 |

Table 9

*Descriptive Statistics for a Number of Indicators of Palatability, Recorded during Acquisition Trails with Sodium Gluconate, Followed by Injections of Lithium Chloride. Data are Separated by Day: Day 1 Indicating the First Exposure to Sodium Gluconate, and Day 2 Indicating the Second Exposure*

|  | Day 1 | | Day 2 | |
|---|---|---|---|---|
|  | Mean | SE | Mean | SE |
| Total Licks | 420.00 | 64.16 | 407.50 | 17.48 |
| Licks/Burst | 17.90 | 2.13 | 17.65 | 1.55 |
| Efficiency | 0.82 | 0.02 | 0.84 | 0.01 |
| MPI | 101.33 | 2.07 | 102.24 | 2.12 |