Published in final edited form as:

Physiol Behav. 2018 October 01; 194: 410–419. doi:10.1016/j.physbeh.2018.06.030.

Altered salt taste response and increased tongue epithelium *Scnna1* expression in adult *Engrailed-2* null mice

Ankita Gupta^a, Xinyi Li^b, Emanuel DiCicco-Bloom^c, and Nicholas T. Bello^{a,b,*}

^aDepartment of Animal Sciences, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA.

^bNutritional Sciences Graduate Program, School of Environmental and Biological Sciences, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA.

^cDepartment of Neuroscience and Cell Biology/Pediatrics, Rutgers Robert Wood Johnson Medical School, Rutgers, The State University of New Jersey, Piscataway, New Jersey, USA

Abstract

Sensory impairments are critical for diagnosing and characterizing neurodevelopmental disorders. Taste is a sensory modality often not well characterized. *Engrailed-2* (*En2*) is a transcription factor critical for neural development, and mice lacking *En2* (*En2*^{-/-}) display signs of impaired social interaction, cognitive processes (e.g., learning and memory, conditioned fear), and neurodevelopmental alterations. As such, *En2*^{-/-} mice display the behavioral deficits and neural impairments characteristic of the core symptoms associated with autism spectrum disorder (ASD). The objective of this study was to characterize the taste function in *En2*^{-/-} compared with *En2*^{+/+} in adult male mice. Measuring taste responsiveness by an automated gustometer, *En2* null mice had decreased lick responses for 1.6 M fructose, whereas they demonstrated an increased taste responsivity (i.e., relative to water) at 0.3 M sodium chloride and 1 M monosodium glutamate. In a separate cohort of mice, *En2*^{-/-} mice had an increased preference for sodium chloride over a range of concentrations (0.032 – 0.3M) compared with *En2*^{+/+} mice. Regional gene expression of the tongue epithelium demonstrated an increase in *Scnn1a*, *T2R140*, *T1R3*, and *Trpm5* and a decrease in *Pkd1l3* in *En2* null mice. Taken together, such data indicate that deficits in *En2* can produce sensory impairments that can have a measurable impact on taste, particularly salt taste.

1. Introduction

Taste is a sensory modality essential for the ingestion of nutrients and hedonic evaluation of food. Developmental events during childhood and adolescence can alter diet selection and affect preference for certain foods [1]. Picky eating or self-restrictive eating patterns are common in developmental disorders, such as autism spectrum disorder (ASD) [2–4]. Indeed, some level of feeding difficulties occurs in as much as 90% of children diagnosed with ASD and other pervasive developmental disorders [3–5]. Although ASD is a neurodevelopmental disorder that is characterized by deficits in socialization and communication, this disorder can encompass a variety of behavioral and emotional responses [2]. Classification of ASD

*Corresponding author, ntbello@rutgers.edu (NTB).

based on the type of sensory impairment could be beneficial for developing phenotype-based treatments [6]. Compared with other sensory modalities, there are few investigations into the basis for the taste impairments associated with ASD [7]. Previous studies analyzing taste identification accuracy have shown that adolescents with ASD have more difficulty identifying aversive taste stimuli [8]. Another study also found that adults with ASD have difficulty identifying sweet and aversive taste stimuli and often mislabel taste stimuli as salty [9]. Notwithstanding, those few tastes studies that have been performed in ASD subjects have not examined the possible role of heritable factors on their findings [8–10].

Although there are several genetic variations and possible candidate genes implicated in ASD, single nucleotide polymorphisms, *rs1861972* (A/G) and *rs181973* (C/G), in the intronic region of the *Engrailed 2* (*EN2*) homeobox gene have been associated with ASD in human populations [11–14]. *Engrailed 2* is a transcription factor that is critical for neural development and affects norepinephrine levels in the forebrain and hindbrain [15]. *En2* knockout (*En2*^{-/-}; KO) mice display signs of poor social interaction and cognitive behaviors that are characteristic of ASD [16]. Further, this mouse model exhibits deficits in learning and memory, deformities in cerebellar development, and a decrease in the number of cerebellar Purkinje cells [12, 17–19]. Our laboratory recently reported that a juvenile exposed (postnatal day 21 to 60) to a ketogenic diet improved the autistic-like social impairments and exploratory behaviors associated with adult *En2* KO mice [20]. Taken together, this suggests the *En2* KO mouse has a behavioral phenotype that can be a useful model for uncovering the sensory deficits associated with ASD.

The following study was designed to understand the taste sensory impairments in the *En2*^{-/-} mouse. Specifically, these experiments examined the taste acceptability and preference for established tastes (i.e., salt, sweet, bitter, sour, and umami). In addition, we examined taste-receptor gene expression of the tongue epithelium, which included *Scnn1a* (salty), *Tas1r1* (umami), *Tas1r3* (sweet and umami), *Tas2r116* (bitter), and *Tas2r140* (bitter), as well as taste cell type and taste bud related genes (*Trpm5*, *Plc-β2*, *Pkd113*, and *Kcnq1*) [21–23]. This is the first study to characterize the taste dysfunction associated with *Engrailed 2* gene status. Thus, understanding the impaired sensory processing in *En2*^{-/-} compared with *En2*^{+/+} (WT; Wildtype) will help to further characterize the sensory impairments associated with autism-related behaviors and associated genes.

2. Materials and Methods

2.1. Mice

En2^{tm1Alj/tm1Alj} mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). These mice were generated on a 129S2/SvPas background as previously described [16]. *En2* heterozygous (*En2*^{+/-}) breeding pairs were placed on a 12:12 h light:dark cycle with lights off at 1800 h. To prevent genetic drift, every ten generations, *En2*^{+/-} mice were crossed to B6129SF2/J mice for creation of new *En2*^{+/-} breeding pairs. Pups were kept with the dam until weaning at postnatal day (PND) 21. After weaning, male juveniles were housed 2–4 mice per cage. All experiments were conducted using adult (PND 56) *En2* WT and KO male littermates. Mice were fed standard chow (Purina Mouse Diet 5015, 25.34% fat, 19.81% protein, 54.86% CHO, 3.7 Kcal/g) and water was available at all times, unless

otherwise noted. Genomic DNA from ear clippings was used to genotype animals, following previously published protocol [20]. All animal work was conducted according to relevant national and international guidelines. Animal care protocol was approved by the Institutional Care and Use Committee at Rutgers University (OLAW #A3262-01, protocol#13-001).

2.2. Taste responsivity assessment

A brief access taste assessment was performed as previously described, but with slight modifications [24, 25]. An automated gustometer (Davis Rig; Dilog Instruments, Tallahassee, Florida) was used to measure the number of licks for the different concentrations of taste stimuli for each animal [24] [26] [27]. During the light cycle (~ 0900 h) the mouse was placed in the plexiglass testing chamber (6"W x 11"H x 10"D) with access to a single spout of water. The floor was composed of a stainless-steel grid, and both the chamber and floor could be sanitized. The chamber was connected to a motor-driven bottle rack that could hold up to 16 bottles at a time. Throughout the study a maximum of 7 bottles were used. Each mouse was given a 5 min acclimatization to the gustometer chamber before initiating a taste session. A session was initiated with the opening of the shutter and presentation of a tube spout with a tube containing a single concentration of a taste stimulus. The concentration was presented for 5 secs. The intertrial interval (ITI) was 8 secs. There were 15 presentations of each tube with a maximum of 105 trials occurring over a period of 25 minutes.

Beginning at PND 63, male mice, cohorts of WT (n = 9) and KO (n = 17), were water restricted for 24 h prior to water training for 4 consecutive days. Mice were only allowed access to water during the training sessions. Specifically, on days 1 and 2 of behavioral water training, mice were allowed free access to 2 bottles of water for 15 min each. On days 3 and 4, each animal was given access to 7 bottles of water for a total of 25 min with a presentation time of 5 sec for each bottle and ITI of 8 sec for a total of 105 trials. After day 4, animals were given water *ad libitum* in their home cages. The first round of taste stimuli sessions started the following week. For the testing sessions, mice were tested with 7 different taste stimuli at 6 different concentrations under a partial caloric and water restriction (See Supplemental Table 1). Mice were individually housed and were given ~ 1 g standard chow and ~ 2 mL water, 24 h prior to each test day. The taste stimuli included: sucrose (sweet disaccharide: 0, 0.01, 0.03, 0.1, 0.3, 1 and 1.5 M), fructose (sweet monosaccharide: 0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 M), sodium chloride (salty: 0, 0.01, 0.03, 0.06, 0.1, 0.3 and 1 M), monosodium glutamate (umami: 0, 0.01, 0.03, 0.06, 0.1, 0.3 and 1 M), alanine (sweet amino acid: 0, 0.01, 0.03, 0.06, 0.1, 0.3 and 1 M), citric acid (sour: 0, 0.001, 0.003, 0.006, 0.01, 0.03, 0.1 M) and quinine-HCL (bitter: 0, 10^{-5} , 3×10^{-5} , 10^{-4} , 3×10^{-4} , 10^{-3} , 3×10^{-3} M). Mice were given an intervening day of chow and water *ad libitum* between taste stimuli. Concentrations were presented in a standardized design to minimize contrast and order effects (i.e., non-ascending/non-descending fashion). All taste stimuli were prepared on the day of the experiment using filtered tap water (see Supplemental Table 2). This was continued daily over a period of 6 weeks (40 days) so that each mouse was exposed to all the taste stimuli twice in the same sequence (Supplemental Table 1 & 2). Only data from the second session were used for the analysis. A total of 2 WT and 1 KO mice were excluded from the data set because they failed to lick any of the taste

stimuli at any concentration during the sessions. We did include in the data set mice that did not lick at a concentration of a taste stimulus, their recorded lick response was “0”.

2.3. Taste preference assessment

In a separate naïve cohort of WT (n = 10) and KO (n = 16) male mice (PND 56 to 70), a two-bottle choice test was performed as previously described but with slight modifications [28]. Mice were individually housed and tested with 4 different taste stimuli at different concentrations. Testing was performed in ascending order. Taste stimuli tested were sodium saccharin (artificial sweetener: 0, 0.0025 and 0.01 M), sodium chloride (salty: 0, 0.032, 0.075, 0.15, 0.3 and 0.6 M), monosodium glutamate (umami: 0, 0.01, 0.032, 0.1, 0.32 and 0.6 M) and quinine-HCl (bitter: 0, 3.2×10^{-5} , 10^{-4} , 3×10^{-4} M).

Animals were allowed access to the two bottles for 48 h. One bottle was filtered tap water and the other contained one concentration of the taste stimuli. After a period of 24 h, the positions of the bottles were switched in order to avoid place preference. Animals were given an intervening day of *ad libitum* water after each concentration of taste stimulus. Percent preference was expressed by taking the volume of taste stimuli consumed divided by the total volume of fluid consumed (taste stimulus and water) multiplied by 100. A taste stimulus with a preference score greater than 50 was considered to be *preferred*, less than 50 was *avoided*, and equal to 50 was *indifferent* relative to water (i.e., no more or less palatable).

2.4. Tissue dissections for quantitative real-time PCR (qPCR)

In a separate cohort of WT (n = 12) and KO (n = 8) male mice (PND 60) tongues were removed following decapitation, transferred to Sorenson's phosphate buffer, and were stored in RNeasy lysis buffer (ThermoFisher Scientific, Inc.) overnight at 4°C. The tongue epithelium was microdissected under a dissection microscope for the circumvallate papillae (CV), foliate papillae (FO), and fungiform papillae (FU) regions following regional delineations [29]. To ensure consistency between regions and sample collections, the same lab member performed all the tongue tissue dissections. The individual performing the dissections was blinded to genotype of the mouse throughout the entire experiment. The dissected tissue was stored in -80°C until RNA extraction. Total RNA was extracted using RNeasy lysis buffer (ThermoFisher Scientific, Inc.) and treated with DNase-I in order to reduce any genomic DNA contamination. RNA quantity and quality were determined using the NanoDrop ND-2000 spectrophotometer (ThermoFisher Scientific, Inc.).

2.5. Quantitative real-time PCR

Reverse transcription was performed on 200 ng/ul RNA using 0.5ul Super Script™ III Reverse Transcriptase (ThermoFisher Scientific, Inc.), 4 µl 5X FS buffer, 1.25 µl dNTP, 1µl 100 ng random hexamer primer, 0.38 µl Rnasin and 2 µl 100mM SS DTT in DEPC treated water (Gene Mate, Bioexpress, Inc.) to synthesize 20 µl of cDNA. Reverse transcription was conducted using a thermocycler according to the following protocol: incubation at 25°C for 5 mins, transcription at 50°C for 60 min, denaturation at 70°C for 15 min and then cooled to 4°C for 5 min. A 1:20 serial dilution was performed on the cDNA using nuclease free water (Gene Mate, Bioexpress, Inc.) and the final concentration was 0.5ng/ul. Samples were stored

at -20°C . CV, FO, and FU regions from female WT mice served as the positive control. FO region from each male KO mouse (no reverse transcriptase) served as the negative control to ensure the cDNA was not contaminated with genomic DNA [30].

All PrimePCR™ primers were purchased from Bio-Rad Laboratories, Inc and are commercially available. These primers included *En2* (Accession Number: NM_010134.3), *Scnn1a* (NM_011324.2), *Tas1r1* (NM_031867.2), *Tas1r3* (NM_031872.2), *Tas2r116* (NM_053212.1), *Tas2r140* (NM_021562.1), *Trpm5* (NM_020277.2), *Plcβ2* (NM_001290790.1), *Pkd113* (NM_001286454.1), and *Kcnq1* (NM_008434.2). Primers of housekeeping genes, *Actb* and *Gapdh*, were synthesized by Life Technologies, Inc. Sequence Primers for *Actb* (Accession Number: NM_007393.5) were GCCCTGAGGCTCTTTTCCA and TAGTTTCATGGATGCCACAGGA and *Gapdh* (Accession Number: NM_008084.3) TGACGTGCCGCTGGAGAAA and AGTGTAGCCCAAGATGCCCTTCAG. qPCR was performed on 0.5 ng/μl cDNA using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) and nuclease free water. Relative mRNA expression was calculated using $\Delta\Delta$ cycle threshold (CT) method. A CFX-Connect real-time PCR instrument was used to amplify the samples according to the following protocol: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 45 sec and extension, as previously described [31]. A final dissociation step was used to melt the samples with 60 cycles of 95°C for 10 sec, 65°C to 95°C (in increments of 0.5°C) for 5 sec and 95°C for 5 sec. Positive, negative and blank water controls were included in each plate design. Tissues that failed to produce sufficient RNA or CT values were excluded from the data set.

2.6. Statistical analyses

For the taste responsivity experiment, mean number of licks was calculated across all trials for a given taste stimulus to create concentration-response curves for each taste stimulus. Additionally, lick numbers were also standardized as either licks relative to water value (e.g., sucrose, fructose, alanine, and monosodium glutamate) or taste stimulus/water lick ratio (e.g., sodium chloride, citric acid, quinine HCl) [24, 32]. The means for each concentration were used to calculate the licks relative to water value: mean number of licks (each concentration of chemical X) – mean number of licks (water) = licks relative to water [24]. For the taste preference experiment, fluid intake and preference scores were calculated. Total fluid intake per day was calculated by averaging the total amount of taste stimulus concentration and water consumed over 48 hours. Water intake was an average of the water consumed during all the testing sessions. Preference scores were calculated by dividing the fluid intake of a specific concentration per day by total fluid intake per day and multiplied by 100% [28]. A repeated-measures analysis of variance (ANOVA) was used to determine genotype, concentration and genotype x concentration effects. Fisher LSD post hoc testing were performed when justified [25, 33, 34]. Planned comparisons were performed when there was a concentration effect and post-hoc significance at an individual concentration. Independent t-tests were used to determine the effects of body weight between genotypes.

For the gene expression, relative mRNA expression was calculated using the Cq method using 1:20 diluted pooled WT female samples ($n = 2$) for each region as the calibrator tissue

[35, 36]. Absorption threshold was set at the lowest point of the exponential curve where the slope of the curve was the steepest for all plates [31]. Linear quantity of target genes was calculated using 2^{-Cq} . Data were expressed as n-fold difference to the WT group. Independent T-tests were performed for individual genes for each tongue region. All statistical analyses were performed using Statistica 7.1 software (StatSoft, Tulsa, OK, USA) and significance was set at $\alpha = 0.05$.

3. Results

3.1. Taste assessment using an automated gustometer

Brief access taste assessments revealed several genotype-dependent differences. Expressing the data as either mean number of licks or standardized licks (i.e, licks relative to water or taste stimulus/water lick ratio) did not change the overall statistical findings. As a result, only the mean number of licks are reported. The recorded response for the mice that did not lick was recorded as “0” and these data are represented in the mean values shown in Fig 1A–G. Alternatively, since a mean value of “0” represents a “no response” over the 15 trials at a taste stimulus concentration, a separate representation of the data with “0” values removed is illustrated in Supplemental Fig 1A–G. Although the differences between the KO and WT are more robust for some concentrations of sodium chloride, citric acid, and quinine HCl in the Supplemental Fig 1C, F and G, using the dataset with “0” values removed limited our ability to appropriately perform the repeated measures analyses. Therefore, we did not eliminate the “0” values from the dataset used for the analyses detailed below. For sucrose, repeated measures ANOVA revealed an effect for concentration [$F(6, 126) = 31.2, p < 0.001$]. There were greater number of licks at 0.3M, 1.0M, and 1.5M sucrose compared with water ($p < 0.001$), see Fig 1A. For fructose, there were effects for concentration [$F(6, 126) = 21.7, p < 0.001$] and genotype X concentration [$F(6, 126) = 2.5, p < 0.05$]. For concentration, there were increased lick rate for fructose at 0.4M, 0.8M, and 1.6M compared with water ($p < 0.001$). The KO group had a lower number of licks for fructose at 1.6 M ($p < 0.05$), see Fig 1B. For sodium chloride, there was an effect for concentration [$F(6, 126) = 2.2, p < 0.05$]. Post-hoc testing revealed significantly greater mean number of licks for NaCl at 0.3M compared water ($p < 0.05$). Planned comparison revealed a difference between KO and WT at 0.3M, with the KO animals having a greater number of licks ($p < 0.05$), see Fig 1C. For monosodium glutamate, there was effect of concentration [$F(6, 126) = 8.9, p < 0.001$]. For concentration, there were increased lick rates for MSG at 0.3M and 1.0M compared with water ($p < 0.001$). Although there was genotype x concentration effect that approached significance [$F(6, 126) = 3.1, p = 0.05$], planned comparisons revealed that the KO mice had significantly greater number of licks at 1.0M compared to the WT cohort ($p < 0.05$), see Fig 1D. For alanine, there was an effect for concentration [$F(6, 126) = 15.3, p < 0.001$]. There were more licks for alanine at 0.3M and 1M compared with water ($p < 0.001$), Fig 1E. There were no differences for citric acid or quinine for mean number of licks, see Fig 1F and 1G. At the completion of the sessions, there were no body weight differences between the two groups (WT = $25.3 \pm 1.5g$, KO = $27.5 \pm 0.8g$).

3.2. Fluid intake and percent preference using a two-bottle test

Two-bottle preference assessments revealed several genotype-dependent differences. Fluid intake and preference scores are shown in Fig 2. For sodium saccharin fluid intake, there was an effect of genotype [$F(1, 24) = 4.6, p < 0.05$] and concentration [$F(2, 48) = 73.9, p < 0.001$]. Post-hoc tests revealed fluid intake was lower in KO ($p < 0.001$). The KO group had significantly lower intake at 0.01 M compared with the WT mice ($p < 0.05$), see Fig 2A. There were not any differences in percent preference for sodium saccharin between the genotypes. However, the mice displayed a preference response for the concentrations of saccharin presented, see Fig 2 B. For sodium chloride fluid intake, there was an effect of concentration [$F(5, 120) = 34.0, p < 0.001$] and genotype x concentration effect [$F(5, 120) = 6.6, p < 0.0005$]. Post-hoc testing revealed that KO mice had lower water (0 M) intake and higher intakes at 0.15M NaCl compared with WT mice ($p < 0.001$), see Fig 2C. For preference, there was a genotype effect [$F(1, 24) = 22.0, p < 0.0001$] and a concentration effect [$F(4, 96) = 23.0, p < 0.0001$]. There were higher preference for NaCl in the KO compared with the WT group at 0.032, 0.075, 0.15 and 0.3M, ($p < 0.05$ for all), see Fig 2D. Thus, the KO mice displayed an indifference-avoidance response, whereas the WT mice displayed an avoidance response for the concentrations of sodium chloride presented. For monosodium glutamate fluid intake, there was only a concentration effect [$F(5, 120) = 5.8, p < 0.001$], see Fig 2 E. There was a concentration effect [$F(4, 96) = 12.9, p < 0.0001$] for percent preference. Both groups of mice displayed an indifference- avoidance response for the concentration of monosodium glutamate presented, see Fig 2F. For quinine HCl fluid intake, there were effects of genotype [$F(1,24) = 6.7, p < 0.05$] and concentration [$F(3,72) = 55.7, p < 0.001$]. Post-hoc testing revealed that KO mice consumed less fluid than WT ($p < 0.05$). The KO mice drank less water (0 M) and 3.2×10^{-4} M quinine compared with WT ($p < 0.05$, for both), see Fig 2 G. For percent preference there was only a concentration effect [$F(2, 48) = 23.6, p < 0.0001$] and displayed at avoidance responds to the concentrations presented, see Fig 2 H. At the completion of the 2-bottle preference tests, there were no body weight differences between the two groups (WT = 31.4 ± 2.9 g, KO = 30.2 ± 1.7 g).

3.3. Relative mRNA expression in distinct tongue regions

There were genotype-dependent differences in the relative gene expression of taste-related genes. For *En2*, relative expression was not measurable in KO mice. *En2* relative expression was lower in the KO group compared to WT for CV, FO, and FU ($p < 0.00001$ for all). For *Scnn1a*, there was higher expression in KO compared with WT groups in the CV [$t(18) = 3.28, p < 0.005$], FO [$t(17) = 5.8, p < 0.005$] and FU [$t(17) = 8.0, p < 0.0001$] regions, see Fig 3 A, D and G. For *TIR3*, there was higher expression in KO compared with WT in the FO [$t(17) = 3.1, p < 0.01$] and FU [$t(16) = 4.7, p < 0.005$] regions, see Fig 3 F and I. For *T2R140*, there was higher expression in the KO compared with WT in the CV region [$t(14) = 2.4, p < 0.05$], see Fig 4 B. For *TRPM5*, there was higher expression in the KO compared with WT in the CV region [$t(13) = 3.05, p < 0.05$], see Fig 4 C. For *Pkd1l3*, KO group had a decreased expression in CV region [$t(12) = -2.3, p < 0.05$], see Fig 5 B. At the time of euthanasia for tissue removal, there were no body weight differences between the two groups (WT = 22.3 ± 0.9 g, KO = 23.8 ± 2.8 g).

4. Discussion

The present study investigated the role of *engrailed 2* (*En2*) gene status on taste sensory processing in male mice. Male mice were used in this study since ASD is more common in male children [37]. Our results indicate that lack of the *En2* gene resulted in measurable impaired taste responses over a limited range of taste stimuli concentrations and altered gene expression of taste related genes in the tongue epithelium. Specifically, we found that *En2* null mice had decreased taste responsivity for 1.6M fructose and increased taste responsivity of 0.3 M sodium chloride and 1 M monosodium glutamate. One limitation was the testing conditions for the brief access taste responsivity test were performed under calorie restriction for our experiments. Typically, only sucrose or other saccharides are tested under calorie restricted conditions [32]. In the present experiments, all taste stimuli, including aversive taste stimuli, were tested under calorie-restricted conditions to ensure motivational performance in the brief-access tests, since *En2* null mice have learning and memory impairments that could potentially affect sipper tube sampling [16]. One possible unintended consequence of the calorie-restricted testing conditions was a lack of a dose-response effect for citric acid and quinine in the taste responsivity test. Notably, the *En2* null mice did have elevated expression of the bitter taste receptor *T2r140* and lower 3×10^{-4} M quinine intake during a two-bottle preference test. These data suggest that *En2* null mice could have minor impairments in bitter and sour taste response that were masked by the calorie deprived testing conditions during the brief taste responsivity test. A limitation of our brief taste responsivity findings, however, was that we did not account for the olfactory sensory properties of the taste stimuli.

En2 null mice had less avoidance for sodium chloride over a range of concentrations (0.032–0.3M). Regional gene expression of the tongue epithelium demonstrated an increase in α subunit of the epithelial sodium channel (*Scnn1a*) in *En2* null mice. Increases in the taste receptor, type 2, member 140 (*T2R140*) in circumvallate region and increase in the taste receptor, type 1, member 3 (*T1R3*) in the foliate and fungiform regions were also observed in *En2* null mice. One limitation of our findings is that the *En2* null mice had a 129S hybrid background. The *En2* null transgene on the 129S background has demonstrated social impairment and deficits associated with ASD [15, 16, 20]. It is well-established that there are strain-dependent differences in the 129 background taste responses and preferences, but what is clear from our findings is that mice lacking *En2* on the 129S hybrid background had quantitative differences in taste outcomes [33, 38, 39]. This hybrid background could be an alternate explanation of the relative flat taste response to citric acid and quinine in the brief access taste assessment test. Nonetheless, the influence of mouse strain background required further investigation. Taken together, such data indicate that in adult male mice the lack of *En2* can produce measurable taste sensory impairments.

Impairments in taste identification has been noted in ASD subjects [7]. Bennetto and colleagues examined taste identification and thresholds in children with ($n = 21$) or without ASD ($n=27$) [8]. Children with ASD were less accurate in identifying citric acid (10%) and marginally less accurate at identifying quinine (0.25%), but there were no differences in the electrogustometry detections between groups [8]. Because taste is a quantifiable sensory modality that can be systematically investigated, uncovering the possible taste impairments

in a mouse model that display autistic-related behaviors was the rationale for this study. Although there are other candidate genes associated with ASD, the *En2* gene encodes a homeobox transcription factor critical for the regionalization of the developing midbrain and cerebellum [40]. In human studies, two polymorphisms, *rs1861972* (A/G) and *rs1861973* (C/G), that are 152 bp apart in the intronic region of *En2* gene have been associated with autism spectrum disorder [12–14, 41]. In a family-based association analysis of 138 triads (both parent and affected child), an over-transmission of the A allele of *rs1861972* (73 of the 113 heterozygous parents) and C allele of *rs1861973* (71 of the 104 heterozygous) was reported. In addition in this population, the *rs1861972* and *rs1861973* A-C haplotype had a frequency of 68.8% and was over-transmitted to ASD-affected offspring [41]. In post-mortem cerebellar tissue from ASD (n = 35) and control (n = 55) subjects, ASD subjects that were heterozygous for the haplotype (*rs1861972* and *rs1861973* A-C) had increased *En2* gene expression [14]. Also, heterozygous ASD had increased gene expression of sonic hedgehog (*SHH*) and individuals heterozygous for the *rs1861972* and *rs1861973* A-C haplotype had increased *SHH* gene expression [14]. In addition, midbrain tissue from *En2* null mice show decreased *SHH* expression, suggest that the ASD-associated *En2* haplotype is a transcription activator [14]. Based on the role of *En2* as a patterning gene for neural development, mice that lack the *En2* gene display various behavioral deficits that have been associated with the social-interactions difficulties and increased repetitive behaviors, which are core symptoms of ASD [2, 16, 20]. Specifically, *En2* null mice have fewer bouts of social reciprocity, lack socialization, and reduced male and female interactions [16, 20]. The present study extends the deficits demonstrated by *En2* null mice to include taste impairments.

Although the *En2* null mice did not display profound differences in taste function compared with *En2* intact mice, *En2* null did have alterations in salt taste. Indeed, *En2* null mice had increased taste responsivity, reduced avoidance of salt, and increased tongue epithelium expression of *Scnn1a*. There was also increased expression of *T1r3*, *Trpm5*, and *T2r140* and decrease expression of polycystic kidney disease-1-like 3 (*Pkd1l3*) in the *En2* null mice. Notably, there was a decrease in the taste responsivity in the brief access test for the highest fructose (1.6M) concentration and reduced intake of the highest saccharin (0.01M) concentration in the 2-bottle test in the *En2* null mice. In a series of 2-bottle preference tests, *T1r3* knockout mice have been shown to consume less 2–32% fructose and less fructose than water at 16–32% concentrations compared with wild type mice [42]. Therefore, the increase expression of the sweet taste subunit, *T1r3*, could reflect a shift in the sweet taste in *En2* null mice. Saccharin also has a bitter component and has known to activate the *Tas2* class of receptors [43], therefore, increased expression of the bitter taste subunit, *T2r140*, could also reflect a shift in saccharin acceptance. The mechanisms of how *En2* is impacting taste is entirely unclear. While engrailed-like proteins have been identified in the taste sensory epithelium of zebrafish [44], our findings indicated *En2* gene expression is evident in the tongue epithelium *En2*^{+/+} mice. One possibility effect on taste function is that *En2* regulates *SHH* in lingual tissue. That is, *SHH* is expressed in undifferentiated primary taste cells and plays a role in taste cell lineage [45, 46]. Prolonged treatment (15-week) with a selective *SHH* pathway antagonist, vismodegib, in adult mice produced alteration in taste bud morphology and expression of taste-related genes, including *T1R3* [47]. In addition,

because *Scnn1a* is a subunit of non-voltage gated sodium channels present in taste buds and other epithelia tissues besides tongue [48], the increase in salt taste and tongue epithelia *Scnn1a* expression could reflect a broader regulation of *En2* on other sodium-dependent homeostatic systems. Considering the expression of *Scnn1a* in other tissues, the metabolic consequence of salt ingestion in the *En2* null mice is an unanswered question. Therefore, it is possible that the post-ingestive metabolic consequences of the sodium chloride during the 48 h two-bottle test influenced taste preference response. Considerable evidence also indicates that *En2* is a candidate oncogene in some types of cancer, and *En2* has been explored as a urinary biomarker for prostate cancer [49, 50]. These data suggest that *En2* could have additional functions beyond that of homeobox transcription factor. Several limitations of our interpretations of *En2* on taste outcomes were that we did not determine if the *En2* null mice had anatomical differences in tongue morphology or if *En2* was expressed specifically in the taste buds in the *En2*^{+/+} mice. Our findings that a taste cell type III marker, *Pkd1l3*, was decreased suggest a regional difference in the distribution and function of taste cells in the *En2* null mice [51]. Another limitation of our findings is that the taste responsivity assessment, taste preference test, and gene expression were performed under different dietary conditions, and it is unclear whether dietary conditions differentially influence taste in *En2* null mice. Moreover, the brief taste responsivity and the two-bottle preference test findings could have been influenced by olfactory cues, which was not controlled in the present studies. Future studies will investigate how dietary manipulate sensory response in *En2* null mice and *En2* influences basal taste cells, taste tissue morphology, and *SHH* in tongue development and taste responses.

5. Conclusions

Sensory dysfunctions, in particular olfactory impairments, have been well-characterized in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Dysfunctions associated with neurodegenerative diseases, however, are typically investigated for their clinical utility as early markers for disease detection, severity, and progression [52]. In contrast, characterization of the sensory dysfunction associated with neurodevelopmental disease and their associated genes can provide some insight into developing effective treatment strategies [6]. The major findings of our study implicate *En2* as a possible modulator of salt taste and salt-related genes in mice. How *En2* gene variants impacts taste and sensory dysfunction in patients diagnosed with autism or related neurodevelopmental remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Drs. Troy A. Roepke and Jessica Verpeut for their technical assistance and Kathy Manger for her editorial assistance. Additional thanks to Ami Patel, Samantha Meza, Thissa Thambugala, Sarah Walsh and Samuel Liu for their assistance with the behavioral experiments. NTB is supported by NIH R01AT008933 and USDA-NIFA NJ06180.

7. References

- [1]. Hoffman AC, Salgado RV, Dresler C, Faller RW, Bartlett C Flavour preferences in youth versus adults: a review. *Tob Control*. 2016;25:ii32–ii9. [PubMed: 27633764]
- [2]. Association AP Diagnostic and statistical manual of mental disorders: DSM-V. 5th ed. Washington, DC: American Psychiatric Association; 2013.
- [3]. Manikam R, Perman JA Pediatric feeding disorders. *J Clin Gastroenterol*. 2000;30:34–46. [PubMed: 10636208]
- [4]. Schreck KA, Williams K Food preferences and factors influencing food selectivity for children with autism spectrum disorders. *Res Dev Disabil*. 2006;27:353–63. [PubMed: 16043324]
- [5]. Ahearn WH, Castine T, Nault K, Green G An assessment of food acceptance in children with autism or pervasive developmental disorder-not otherwise specified. *J Autism Dev Disord*. 2001;31:505–11. [PubMed: 11794415]
- [6]. Lane AE, Molloy CA, Bishop SL Classification of children with autism spectrum disorder by sensory subtype: a case for sensory-based phenotypes. *Autism Res*. 2014;7:322–33. [PubMed: 24639147]
- [7]. Boudjarane MA, Grandgeorge M, Marianowski R, Misery L, Lemonnier E Perception of odors and tastes in autism spectrum disorders: A systematic review of assessments. *Autism Res*. 2017.
- [8]. Bennetto L, Kuschner ES, Hyman SL Olfaction and taste processing in autism. *Biol Psychiatry*. 2007;62:1015–21. [PubMed: 17572391]
- [9]. Tavassoli T, Baron-Cohen S Taste identification in adults with autism spectrum conditions. *J Autism Dev Disord*. 2012;42:1419–24. [PubMed: 22006402]
- [10]. Damiano CR, Aloï J, Burrus C, Garbutt JC, Kampov-Polevoy AB, Dichter GS Intact Hedonic Responses to Sweet Tastes in Autism Spectrum Disorder. *Research in autism spectrum disorders*. 2014;8:230–6. [PubMed: 24563662]
- [11]. Wang L, Jia M, Yue W, Tang F, Qu M, Ruan Y, et al. Association of the ENGRAILED 2 (EN2) gene with autism in Chinese Han population. *Am J Med Genet B Neuropsychiatr Genet*. 2008;147B:434–8. [PubMed: 17948901]
- [12]. Benayed R, Choi J, Matteson PG, Gharani N, Kamdar S, Brzustowicz LM, et al. Autism-associated haplotype affects the regulation of the homeobox gene, ENGRAILED 2. *Biol Psychiatry*. 2009;66:911–7. [PubMed: 19615670]
- [13]. Benayed R, Gharani N, Rossman I, Mancuso V, Lazar G, Kamdar S, et al. Support for the homeobox transcription factor gene ENGRAILED 2 as an autism spectrum disorder susceptibility locus. *Am J Hum Genet*. 2005;77:851–68. [PubMed: 16252243]
- [14]. Choi J, Ababon MR, Soliman M, Lin Y, Brzustowicz LM, Matteson PG, et al. Autism associated gene, engrailed2, and flanking gene levels are altered in post-mortem cerebellum. *PLoS One*. 2014;9:e87208. [PubMed: 24520327]
- [15]. Genestine M, Lin L, Durens M, Yan Y, Jiang Y, Prem S, et al. Engrailed-2 (En2) deletion produces multiple neurodevelopmental defects in monoamine systems, forebrain structures and neurogenesis and behavior. *Hum Mol Genet*. 2015;24:5805–27. [PubMed: 26220976]
- [16]. Brielmaier J, Matteson PG, Silverman JL, Senerth JM, Kelly S, Genestine M, et al. Autism-relevant social abnormalities and cognitive deficits in engrailed-2 knockout mice. *PLoS One*. 2012;7:e40914. [PubMed: 22829897]
- [17]. Millen KJ, Wurst W, Herrup K, Joyner AL Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants. *Development*. 1994;120:695–706. [PubMed: 7909289]
- [18]. Kuemerle B, Zanjani H, Joyner A, Herrup K Pattern deformities and cell loss in Engrailed-2 mutant mice suggest two separate patterning events during cerebellar development. *J Neurosci*. 1997;17:7881–9. [PubMed: 9315908]
- [19]. Cheh MA, Millonig JH, Roselli LM, Ming X, Jacobsen E, Kamdar S, et al. En2 knockout mice display neurobehavioral and neurochemical alterations relevant to autism spectrum disorder. *Brain Res*. 2006;1116:166–76. [PubMed: 16935268]

- [20]. Verpeut JL, DiCicco-Bloom E, Bello NT Ketogenic diet exposure during the juvenile period increases social behaviors and forebrain neural activation in adult Engrailed 2 null mice. *Physiol Behav.* 2016;161:90–8. [PubMed: 27080080]
- [21]. Mueller KL, Hoon MA, Erlenbach I, Chandrashekar J, Zuker CS, Ryba NJ The receptors and coding logic for bitter taste. *Nature.* 2005;434:225–9. [PubMed: 15759003]
- [22]. Chandrashekar J, Kuhn C, Oka Y, Yarmolinsky DA, Hummler E, Ryba NJ, et al. The cells and peripheral representation of sodium taste in mice. *Nature.* 2010;464:297–301. [PubMed: 20107438]
- [23]. Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, et al. The receptors for mammalian sweet and umami taste. *Cell.* 2003;115:255–66. [PubMed: 14636554]
- [24]. Li X, Treesukosol Y, Moghadam A, Smith M, Ofeldt E, Yang D, et al. Behavioral characterization of the hyperphagia synphilin-1 overexpressing mice. *PLoS One.* 2014;9:e91449. [PubMed: 24829096]
- [25]. Hajnal A, Covasa M, Bello NT Altered taste sensitivity in obese, prediabetic OLETF rats lacking CCK-1 receptors. *Am J Physiol Regul Integr Comp Physiol.* 2005;289:R1675–86. [PubMed: 16081877]
- [26]. Treesukosol Y, Bi S, Moran TH Overexpression of neuropeptide Y in the dorsomedial hypothalamus increases trial initiation but does not significantly alter concentration-dependent licking to sucrose in a brief-access taste test. *Physiol Behav.* 2013;110–111:109–14.
- [27]. Smith JC The history of the “Davis Rig”. *Appetite.* 2001;36:93–8. [PubMed: 11161349]
- [28]. Tordoff MG, Aleman TR, Ellis HT, Ohmoto M, Matsumoto I, Shestopalov VI, et al. Normal Taste Acceptance and Preference of PANX1 Knockout Mice. *Chem Senses.* 2015;40:453–9. [PubMed: 25987548]
- [29]. Kist R, Watson M, Crosier M, Robinson M, Fuchs J, Reichelt J, et al. The formation of endoderm-derived taste sensory organs requires a Pax9-dependent expansion of embryonic taste bud progenitor cells. *PLoS Genet.* 2014;10:e1004709. [PubMed: 25299669]
- [30]. Yang JA, Mamounis KJ, Yasrebi A, Roepke TA Regulation of gene expression by 17beta-estradiol in the arcuate nucleus of the mouse through ERE-dependent and ERE-independent mechanisms. *Steroids.* 2016;107:128–38. [PubMed: 26768413]
- [31]. Gotthardt JD, Verpeut JL, Yeomans BL, Yang JA, Yasrebi A, Roepke TA, et al. Intermittent Fasting Promotes Fat Loss With Lean Mass Retention, Increased Hypothalamic Norepinephrine Content, and Increased Neuropeptide Y Gene Expression in Diet-Induced Obese Male Mice. *Endocrinology.* 2016;157:679–91. [PubMed: 26653760]
- [32]. Glendinning JI, Gresack J, Spector AC A high-throughput screening procedure for identifying mice with aberrant taste and oromotor function. *Chem Senses.* 2002;27:461–74. [PubMed: 12052783]
- [33]. Bachmanov AA, Beauchamp GK Amino acid and carbohydrate preferences in C57BL/6ByJ and 129P3/J mice. *Physiol Behav.* 2008;93:37–43. [PubMed: 17764708]
- [34]. Tordoff MG, Ellis HT Taste dysfunction in BTBR mice due to a mutation of *Itpr3*, the inositol triphosphate receptor 3 gene. *Physiol Genomics.* 2013;45:834–55. [PubMed: 23859941]
- [35]. Pfaffl MW A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001;29:e45. [PubMed: 11328886]
- [36]. Livak KJ, Schmittgen TD Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 2001;25:402–8. [PubMed: 11846609]
- [37]. Wing L Sex ratios in early childhood autism and related conditions. *Psychiatry Res.* 1981;5:129–37. [PubMed: 6945608]
- [38]. Johnson AW, Sherwood A, Smith DR, Wosiski-Kuhn M, Gallagher M, Holland PC An analysis of licking microstructure in three strains of mice. *Appetite.* 2010;54:320–30. [PubMed: 20006663]
- [39]. Boughter JD Jr., Baird JP, Bryant J, St John SJ, Heck D C57BL/6J and DBA/2J mice vary in lick rate and ingestive microstructure. *Genes, brain, and behavior.* 2007;6:619–27.
- [40]. Sgaier SK, Lao Z, Villanueva MP, Berenshteyn F, Stephen D, Turnbull RK, et al. Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrailed proteins. *Development.* 2007;134:2325–35. [PubMed: 17537797]

- [41]. Gharani N, Benayed R, Mancuso V, Brzustowicz LM, Millonig JH Association of the homeobox transcription factor, ENGRAILED 2, 3, with autism spectrum disorder. *Mol Psychiatry*. 2004;9:474–84. [PubMed: 15024396]
- [42]. Zukerman S, Glendinning JI, Margolskee RF, Sclafani A Impact of T1r3 and Trpm5 on carbohydrate preference and acceptance in C57BL/6 mice. *Chem Senses*. 2013;38:421–37. [PubMed: 23547138]
- [43]. Kuhn C, Bufo B, Winnig M, Hofmann T, Frank O, Behrens M, et al. Bitter taste receptors for saccharin and acesulfame K. *J Neurosci*. 2004;24:10260–5. [PubMed: 15537898]
- [44]. Hatta K, Bremiller R, Westerfield M, Kimmel CB Diversity of expression of engrailed-like antigens in zebrafish. *Development*. 1991;112:821–32. [PubMed: 1682127]
- [45]. Miura H, Kusakabe Y, Harada S Cell lineage and differentiation in taste buds. *Arch Histol Cytol*. 2006;69:209–25. [PubMed: 17287576]
- [46]. Miura H, Kusakabe Y, Sugiyama C, Kawamatsu M, Ninomiya Y, Motoyama J, et al. Shh and Ptc are associated with taste bud maintenance in the adult mouse. *Mech Dev*. 2001;106:143–5. [PubMed: 11472844]
- [47]. Yang H, Cong WN, Yoon JS, Egan JM Vismodegib, an antagonist of hedgehog signaling, directly alters taste molecular signaling in taste buds. *Cancer Med*. 2015;4:245–52. [PubMed: 25354792]
- [48]. Hanukoglu I, Hanukoglu A Epithelial sodium channel (ENaC) family: Phylogeny, structure-function, tissue distribution, and associated inherited diseases. *Gene*. 2016;579:95–132. [PubMed: 26772908]
- [49]. Morgan R, Boxall A, Bhatt A, Bailey M, Hindley R, Langley S, et al. Engrailed-2 (EN2): a tumor specific urinary biomarker for the early diagnosis of prostate cancer. *Clin Cancer Res*. 2011;17:1090–8. [PubMed: 21364037]
- [50]. McGrath SE, Michael A, Morgan R, Pandha H EN2 in Prostate Cancer. *Adv Clin Chem*. 2015;71:47–76. [PubMed: 26411411]
- [51]. Yamamoto K, Ishimaru Y, Ohmoto M, Matsumoto I, Asakura T, Abe K Genetic tracing of the gustatory neural pathway originating from Pkd113-expressing type III taste cells in circumvallate and foliate papillae. *J Neurochem*. 2011;119:497–506. [PubMed: 21883212]
- [52]. Field T Smell and Taste Dysfunction as Early Markers for Neurodegenerative and Neuropsychiatric Diseases. *Journal of Alzheimer's Disease & Parkinsonism* 2015;5:1–9.

Fig. 1. Taste responsivity concentration response curves for taste stimuli in adult male *En2*^{+/+} and *En2*^{-/-} mice.

Data were collected using an automated gustometer. A single trial with an individual concentration began with an opened shutter and 5 sec access to the drinking spout. The intertrial interval was 8 secs. There was a maximum of 105 spout-exposures trials for each taste stimulus. The mean number of licks for each concentration for each mouse is the mean value for 15 trials. Water is 0M on the x-axis. The taste responsivity for A: sucrose, B: fructose, C: sodium chloride, D: monosodium glutamate, E: alanine, F: citric acid, and G: quinine in *En2*^{+/+} (WT; closed squares, n = 7) and *En2*^{-/-} (KO; open circles, n = 16) mice.

* indicates $p < 0.05$ post-hoc testing differences from WT for an individual concentration. # indicates $p < 0.05$ planned comparison testing differences from WT for an individual concentration.

Fig 2. Two bottle preference tests for taste stimuli in adult male *En2*^{+/+} and *En2*^{-/-} mice. Fluid intake was recorded at each concentration for 48 h and the bottle order in the cage was switched at 24 h. Intake is expressed as mean 24 h intake (mL/day). Taste stimuli were presented in ascending concentrations with an intervening rest day. Average water intake (0M) for all concentrations of a specific taste stimulus is included in A, C, E and G. Fluid intake is for A: saccharin, C: sodium chloride, E: monosodium glutamate, and G: quinine. Percent preference was also calculated for these taste stimuli by taking the volume of taste stimulus divided by the total volume of fluid (taste stimulus and water) multiplied by 100.

Preference scores are for **B**: saccharin, **D**: sodium chloride, **F**: monosodium glutamate, and **H**: quinine. Dotted line at 50 represents equal preference for water (0M). Intakes were collected in a separate naïve cohort of *En2*^{+/+} (WT; closed squares, n = 10) and *En2*^{-/-} (KO; open circles, n = 16) mice. * indicates $p < 0.05$ post-hoc testing differences from WT for individual concentrations.

Fig 3. Relative gene expression in the tongue regions in adult male *En2*^{+/+} and *En2*^{-/-} mice for *Scnn1a*, *T1R1*, and *T1R3*.

The circumvallate papillae (CV), foliate papillae (FO), and fungiform papillae (FU) tongue epithelia regions were collected in a separate naïve cohort of *En2*^{+/+} (WT; closed squares) and *En2*^{-/-} (KO; open circles) mice. Data was expressed as relative expression calculated using $\delta\delta$ cycle threshold (CT) method and as a n-fold difference relative to the mean of the WT group. **A:** *Scnn1a* expression (WT = 12, KO = 8) in the CV, **B:** *T1R1* expression (WT = 11, KO = 7) in the CV, **C:** *T1R3* expression (WT = 12, KO = 7) in the CV, **D:** *Scnn1a* expression (WT = 11, KO = 8) in the FO, **E:** *T1R1* expression (WT = 9, KO = 4) in the FO, **F:** *T1R3* expression (WT = 12, KO = 7) in the FO, **G:** *Scnn1a* expression (WT = 12, KO = 7) in the FU, **H:** *T1R1* expression (WT = 11, KO = 6) in the FU, **I:** *T1R3* expression (WT =

12, KO = 6) in the FU. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.005$, and **** indicates $p < 0.0001$ between WT and KO for the individual genes in each region.

Fig 4. Relative gene expression in the tongue regions in adult male $En2^{+/+}$ and $En2^{-/-}$ mice for $T2R116$, $T2R140$, and $TRPM5$.

The circumvallate papillae (CV), foliate papillae (FO), and fungiform papillae (FU) tongue epithelia regions were collected in a separate naïve cohort of $En2^{+/+}$ (WT; closed squares) and $En2^{-/-}$ (KO; open circles) mice. Data was expressed as relative expression calculated using $\delta\delta$ cycle threshold (CT) method and as a n-fold difference relative to the mean of the WT group. **A:** $T2R116$ expression (WT = 10, KO = 4) in the CV, **B:** $T2R140$ expression (WT = 10, KO = 6) in the CV, **C:** $TPRM5$ expression (WT = 9, KO = 6) in the CV, **D:** $T2R116$ expression (WT = 7, KO = 4) in the FO, **E:** $T2R140$ expression (WT = 6, KO = 4)

in the FO, **F:** *TRPM5* expression (WT = 5, KO = 4) in the FO, **G:** *T2R116* expression (WT = 4, KO = 4) in the FU, **H:** *T2R140* expression (WT = 4, KO = 4) in the FU, **I:** *TRPM5* expression (WT = 5, KO = 4) in the FU. * indicates $p < 0.05$ and ** indicates $p < 0.01$ between WT and KO for the individual genes in each region.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig 5. Relative gene expression in the tongue regions in adult male *En2*^{+/+} and *En2*^{-/-} mice for *Plcβ2*, *Pkd113*, and *Kcnq1*.

The circumvallate papillae (CV), foliate papillae (FO), and fungiform papillae (FU) tongue epithelia regions were collected in a separate naïve cohort of *En2*^{+/+} (WT; closed squares) and *En2*^{-/-} (KO; open circles) mice. Data was expressed as relative expression calculated using $\delta\delta$ cycle threshold (CT) method and as a n-fold difference relative to the mean of the WT group. **A:** *Plcβ2* expression (WT = 8, KO = 6) in the CV, **B:** *Pkd113* expression (WT = 8, KO = 6) in the CV, **C:** *Kcnq1* expression (WT = 8, KO = 7) in the CV, **D:** *Plcβ2* expression (WT = 9, KO = 6) in the FO, **E:** *Pkd113* expression (WT = 7, KO = 5) in the FO,

F: *Kcnq1* expression (WT = 7, KO = 4) in the FO, **G:** *Plcβ2* expression (WT = 6, KO = 6) in the FU, **H:** *Pkd1l3* expression (WT = 4, KO = 4) in the FU, **I:** *Kcnq1* expression (WT = 9, KO = 6) in the FU. * indicates $p < 0.05$ between WT and KO for the individual genes in each region.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript