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Effects of Proline and Glycine on the Cnidocyte Discharge of Hydra magnipapillata

Janine R. Appleton
University of New Hampshire

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Thesis Advisor: Dr. David Plachetzki

University of New Hampshire

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Abstract

The sense of taste enables animals to utilize environmental cues to detect favorable foods. Through specialized sensory receptors, Cnidarians employ stinging cells called cnidocytes to perform a variety of activities such as locomotion, capturing prey, inducing of feeding responses, and defense. Their discharge is highly regulated by mechanical and chemical signals that are mediated by a complex system including the opsin and taste pathways. Taste 1 Receptors (T1R) have previously been isolated in vertebrates but only until recently, have been noted in invertebrates. Receptors specific to L- amino acids corresponding to the taste sensation of umami, were studied to determine if the pathways of *Hydra magnipapillata* used for feeding were similar to the systems utilized in vertebrates. Amino acids, Proline and Glycine, were experimented using cnidocyte assays to induce feeding and capture cnidocytes. An optimal concentration of 10mM of Proline and Glycine was tested and found to be significant by eliciting greater cnidocyte discharge as compared to a Control of gelatin with P- Values of 0.003 and 0.0011 respectively. This indicates that amino acids, which have similar receptors in vertebrates, are capable of inducing feeding responses in invertebrates implying that T1Rs operate in similar mechanisms thus predating the current notion of the evolutions and diversification of such genes by around 400 million years.

Introduction

Taste 1 Receptors

The taste 1 receptor (T1R) family of G-protein-coupled receptors (GPCR) consists of three different subunits, T1R1, T1R2 and T1R3 all functioning to detect a range of sweetness. These taste receptors operate as chemoreceptors interacting with ligands or other taste stimuli to produce a taste perception in the brain. The T1Rs bind to G proteins, usually gustuctin Gα but also Gαo and Gαi, which activate and inhibit adenylyl cyclases and thus regulate cyclic adenosine monophosphate and cyclic guanosine monophosphates (cGMP) levels[2]. These subparts combine into heterodimers as they are not functional independently. The heterodimer T1R2-T1R3 works in conjunction with the G-protein gustducin, to act as a sweet sense while the heterodimer T1R1- T1R3 senses more a natural sweetness and savor expressing the umami sensations of monosodium glutamate (MSG) [18].

Taste cells in vertebrates are often small biopolar cells that have a relatively short lifetime of 10 days[8]. The cells are packed densely together in groups of 50-150 including precursor cells, support cells, and taste receptor cells[21]. The cells are generally located on the tongue within papillae, the palate, and the pharynx with distinction per type of receptor. The T1R1+3 receptors are found specifically in the fungiform papillae on the tongue and the palate on the roof of the mouth. The T1R2+3 receptors on the other hand, are found in the circumvallate papillae and foliate papillae on the back of the tongue and on the palate on the roof of the mouth[2]. When activated, the T1R1+3 taste receptors synapse on the chorda tympani nerves sending signals to the brain while the T1R2+3 also acts on the glossopharyngeal nerves[2].

Physiology of Taste T1Rs

Taste receptor cells are innervated by nerves that transmit information through the synapse to the brain in order to develop of sort of taste perception[21]. The signal passes through the basolateral side of taste cells via axonal membranes consisting of voltage gated Na^+, K^+, and Ca^{2+} channels[17]. The ligands bind to surface chemoreceptors to activate the GTP- binding protein which causes either Gα to induce the cAMP production via adenylate cyclase activation further activating protein kinase A to initiate actin polymerization and elongation of sensory cells or the activate Gαi to inhibit the cAMP pathway by decreasing adenylate cyclase activity[1,5]. After this chemical stimulation and GPCR activation, the channels become depolarized raising Ca^{2+} levels allowing for action potentials to release through the afferent axons and transmission to occur[17]. The linking of sensory outputs has been recently under investigation. The visual perception of animals is mediated by an opsin-mediated phototransduction cascade. In this process, GPCR signaling pathways are regulated by the depolarization or polarization of ion channels[11].

Umami as a Sense

Umami is related to MSG signifying savor or tastiness represented by the heterodimer of T1R1+3[10]. As it interacts with L- amino acids, it becomes very sensitive to glutamate, inosine monophosphate (IMP), and guanosine monophosphate (GMP) which act to enhance many of the amino acids[8]. The umami sensation is unique from other taste senses as it contains a truncated form of brain mGluR4 which is a metabotropic GPCR originating from the central nervous system. The receptor became adaptive to the high glutamate concentrations from food linking its association to the ribonucleoutides[11]. L-glutamate helps to facilitate the intake of peptides, specifically CCKs, and proteins further heightened by the presence of purines[8].

Animals and Taste

The sense of taste is a necessity for animals as it allows them to systematically pick out food that yields nutritional benefits to them. Most animals respond to food via chemoreception with a lot of focus spent on the effects of amino acids on the stimulation of feeding in aquatic animals. This was first introduced by Nagle in 1802 who proved that dead objects were rejected by sea anemones but objects saturated with food were willingly ingested[16]. Furthermore, a study conducted by Pantin in 1942 tested a variety of food extracts, amino acids, proteins, carbohydrates, and fatty acids on their ability to sensitize the discharges of cnidae. Pantin found that minor discharge rates occurred with just mechanical stimulation, and in order to achieve optimal discharge rates, both chemical and mechanical stimulation was required[19]. The concept that objects needed to be infused with food in order to elicit a response indicated that the stimulus was more sensory related than strictly impulse. This was further studied in a groundbreaking study performed by Loomis, who analyzed the feeding responses of *Hydra littoralis* to glutathione. He found that interaction with glutathione resulted in contraction and writhing of the tentacles and as the concentration of glutathione present increased, the probability that the hydra's tentacles would retract pulling food in toward its mouth also increased[4].

It has been noted in studies that there is a relationship with different chemoreceptors systems in one organism where one amino acid may control the movement of the tentacles while another may control the ingestion of food[20]. The synaptic input to the cnidocytes following stimulus of food implies that sensory nerve cells may serve to detect such stimulus and sensitize the cnidocyte prior to mechanical stimulus and that chemical and mechanical cues from prey help to regulates feeding responses[16, 20].

The Phylogeny of T1Rs in Cnidaria

The presence of T1R in Cnidaria was previously thought to be non- existent due to the absence the receptors during the branching of chordates. Recent phylogenomic analysis however, has since determined that T1R are in fact present in the cnidarian genomes[14].Plachetzki's ability to determine that differing light levels affect the discharge of cnidocytes links the opsin pathways to cnidocytes and thus mechanical and chemical cues[14]. Plachetzki was able to determine that the opsin in the sensory neurons connect to the cnidocytes as well as being complemented by the presence of the cyclic nucleotide gated ion channels which are required for signal transmission[123]. The discovery that cyclic nucleotides channels are the ancestral state of the opsin- mediated photosensitivity help to prove that hydra possess functional opsins despite not having eyes[12]. This suggests that the T1Rs were present prior to the Cambrian Explosion and lost independently thereafter during the lineage diversification of protostomes and tunicates[12].

Cnidocyte Release in Hydra

Hydra are Cnidaria that have been evolving for over 600 million years producing asexually through budding and acting as clones to one another. Hydra are the most primitive organism to have a nervous system with neurons dispersed within the epithelial layers to form networks and bidirectional synapses with non- nervous cells[6, 15]. Organized by radial symmetry, hydra have a mouth encircled by tentacles lined with poison filled cnidocytes. Cnidocytes are found exclusively in Cnidaria and represent one the most complex cell types known in animals[19]. When stimulated, the cnidocytes expel an energetically expensive secretion product called cnidae

in as fast as three milliseconds that are used for multiple operations like feeding, locomotion, and defense[1,3].

The cnidae discharge is under the direct control of the cnidocyte acting as a specialized secretory doubling as a sensory cell with elaborate communication with the nervous system[19]. There are five steps to Cnidarian feeding responses which include: contact of prey to a tentacle, cnidocyte release, contraction of tentacles to the mouth, the opening of the mouth, and finally, ingestion of the prey[9]. The bodily fluids released from the prey after an attack consist of certain chemicals that match receptors on the predator to attract or detract them and elicit feeing reflexes[9].

The cnidae can be discharged due to a variety of reasons such as responding to mechanical stimuli with or without chemosensitization and also due to vibrational frequencies[3]. The discharge is a multi-cellular occurrence requiring both the cnidocyte and surrounding cells, together called the cnidocyte supporting cell complex. This episode is mainly driven by the high pressurized system within the capsule showing the mechanisms acts a sensory neuron[3, 5]. Stimulation of the tentacles causes the tertiary structure of the inactive protein on the surface of cells to alter, activating the protein and thus eliciting a depolarizing effect as the surplus of calcium ions stored in the capsule get released through the opening of the ion channels[5,7]. The concentration gradient yields an influx of water and thus pushes out the cnidae onto the prey.

Plachetzki Laboratory

Research in this laboratory is centered on determining the evolution of sensory genes in Cnidarians, specifically *Hydra magnipapillata*. Dr. Plachetzki's previous work determined that the role of photosensitivity in hydra and more specifically, that hydra, an eyeless organism, have

the ability to use visionary cues through the use of the cyclic nucleotide gated channels. This proved that the opsin genes used for vision in humans may also be attributed to phototransduction cascades in invertebrates. Other members of this lab work to analyze and isolate the opsin pathways within cnidocytes and surrounding cells to determine if taste and vision follow the same transduction cascades. Through double in situ hybridization and cloning of specific sensory genes, the laboratory will be about to use fluorescent markers to localize and show overlap of cell types within the hydra. This research helps to determine if the opsin pathways mediate cnidocyte discharge and thus how cnidarians are able to sense their environments.

Experiment and Expectations

In this study, it was hypothesized that amino acids, specifically Proline and Glycine, were capable of inducing feeding responses as dictated by the T1Rs in Hydra. Both chemical and mechanical stimulation were utilized to determine reflexes with an analysis based quantitatively on the cnidocyte assay and number of probes associated with the desired responses. Compared to a control group, the amino acid coated probes were expected to elicit greater cnidocyte discharge rates. Since vertebrates have a receptor for these amino acids, a behavioral response from the Hydra to Proline and Glycine would indicate that the feeding mechanisms in invertebrates operate similarly to vertebrates undertaking comparable pathways of T1Rs. This would construct a hypothesis on the evolution of T1Rs in invertebrates predating the current understandings of the evolutions and diversification of such genes by around 400 million years.

Materials and Methods

Handling and Preparation of the Experimental Animals

Specimens of *Hydra magnipapillata* in this laboratory are asexual and reproduce via budding. *H. magnipapillata* were kept in glass bowls containing hydra medium (1M $CaCl_2$, 80mM $MgSO_4$, 100mM $MgCl_2$, 1.5M $NaHCO_3$, 30mM KNO_3) and were washed and fed every day. The *H. magnipapillata* were fed Artemia, a salt water- grown marine invertebrate, following standard protocols post washing with new hydra medium. The placement of the Hydra in the laboratory was exposed to the natural daylight cycle while the temperature was regulated to maintain 23°C. In preparation for experimentation, 6-9 plastic FisherBrand polystyrene 100mm X 15mm Petri dishes were obtained and filled halfway with hydra medium. About 9-12 *H. magnipapillata* were removed from the holding dishes and placed in the new Petri dishes via a plastic pipette. The dishes were covered and the *H. magnipapillata* were left untouched and unfed for 24-30 hours. After the allotted time, the Petri dishes were moved to the dark room to be left untouched for 45 minutes.

Preparation of the Solution Probes

Knox unflavored gelatin was obtained as well as Craftsmart Plastalina Modeling Clay. To act as a control for the experiment, 20mg of gelatin was measured with the Sartorius weigh scale and added to a USA Scientific 15mL concial screw cap centrifuge tube to be mixed with 1000mL of hydra medium. L- Proline and Glycine were acquired from Sigma Aldrich and Fisher Scientific respectively. Each amino acid was mixed with milliQ water to produce a stock solution following standard procedures. For preparation of the experiment, variations of milliliters of Proline were used, specifically 1µl, 10µl and 100µl of the stock solution, and were

mixed with 999uL, 990uL and 900uL respectively of hydra medium with Eppendorf pipets and pipet tips as well as with 20mg of gelatin to produce a 1mM, 10mM, and 100mM solution in a USA scientific 15mL centrifuge tube. The second experiment utilized 10µl Glycine mixed with 990µl hydra medium and 20mg of gelatin producing a 10mM solution.

Preparation of the Probes

The Eppendorf Thermomixer was turned on and heated to 36°C. Both the control and experimental tubes were then placed in holders of the Thermomixer and left to liquefy with periodic mixing for 15 minutes. The probes were made from Lebco Omniflex Monofilament fishing line which was 0.008 inches in diameter and a 4lb test weight. A range of 36- 72 pieces of fishing line was cut at 2-3 inches per piece. Depending on the number of hydra being tested, half to one third of the probes were dipped in the into the 36°C control of gelatin and the other half and thirds were dipped into the 36°C amino acid containing tubes. The procedure for dipping consisted of raising and lowering the probes in the solutions three times slowly to minimize the occurrence of air bubbles. Large Petri dishes with a long rod of rolled clay centered in the middle held the dipped probes elevated to prevent drip and surface adhesion. Each tube was maintained at the 36°C temperature range throughout the process. After one round of dipping, the Thermomixer temperature was lowered to 32°C and left for 15 minutes in order for the solutions cool. The control and amino acid based probes were then re-dipped in the respective tubes with the same procedure as before and placed in the dishes and left to dry with the tops on for two hours.

The Cnidocyte Assay and Probing Mechanisms

Three trials were performed with the gelatin- coated control probes and the varying concentrations of solubilized amino acids in gelatin- coated probes. Each trial consisted of six-eight hydra individuals that were probed to trigger cnidocyte discharge. Two sets of 3-4 probes were wet mounted on a FisherBrand Microscope Slide coated with three drops of Amresco Glycerol and topped with a Corning Cover Glass to ease readability and lessen overlap. The probing mechanism consisted of identifying individual hydra that had lengthened and extended tentacles to ease the entry and exit points prior and post contraction of the probes thus limiting attachment of the hydra to the probe. Each individual's distal tentacle was grazed with the distal end of the gelatin coated probe for a count of one. The probe would only be mounted if a) the hydra contracted immediately after probing b) multiple tentacles were not touched in passing and c) the hydra did not attached to the probe. Rotations of trial one would begin with the control and followed by the amino acid dipped probes each utilizing a different Petri dish of hydra. Upon completion, commencement of the second and third trials would occur in the same fashion. Preparation of the experiments was performed in the mornings while counting procedures were performed in the afternoon to evening settings of each day however, it was verified that the light settings remained consistent throughout the research.

Microscopy

The wet mounts and resulting discharged cnidocytes were analyzed under the Differential Interference Contrast (DIC) microscopy. The probes were counted with a LEICA DM 2500 microscope at 10X with follow-ups done at 40X. The analysis accounted for the full length and width of the probe requiring appropriate manual focusing to get a full view of cnidocytes present. Probes were recorded even if no cnidocytes were present however, statistical data does exclude probes with zero values.

Results

Recording of Data

The discharge of cnidocytes in *Hydra* are dictated by both chemical and mechanical cues from the environment ranging from responses due to phototaxis, light exposure, feeding mechanisms and roles of defense as driven by the opsin- mediated phototransduction cascade. A contraction of the body of the *Hydra* into a ball can indicate both adverse and favorable discharge trends. In order to test hypothesis of the optimal concentration of amino acid that would elicit a response to feeding, a series of cnidocyte assays were conducted that differed with amino acids and concentration.

The cnidocyte assay and statistical data accounted for a) probes that had at least one discharged cnidocyte attached b) probes that did not result in hydra attachment post probing c) probes that resulted in hydra contraction post touching the tentacles and d) probes that only touched one tentacle in the process of probing in both the control, Proline, and Glycine solutions. A Fisher's Test, a two tailed T- Test, a Mann- Whitney test, and a Chi- squared test were run for each group, the control and amino acid as well as a comparison of differing amino acid concentrations, to determine if a difference in interaction amounted. A P- Value of less than 0.05 for all tests was considered significant.

Data Collection of Proline

Of the 630 probes made, 458 probes were mounted and 294 probes were counted representing about a 46% utilization rate which is further shown in Table 1. The preliminary results suggested that the optimal concentration of Proline to encourage cnidocyte release was

10mM while 1mM and 100mM matched values of the control test indicating a potential to act as an adverse reactant. The average cnidocyte discharge was highest among amino acids with concentrations of 10mM as a compared to the control group. This was later concurred with statistical data such as the Fisher test (P- Value= 0.0003), referenced in Table 2, Mann- Whitney Test (P- Value = 0.0047), the two tailed T- Test (P- Value= .011), and finally the Chi- square test (P- Value= .000203). This was compared to the 1mM and 100mM and control experiments all of which were not significant (Table 1, Appendix**)**.

Table 1: Statistical Analysis of Proline

	Proline Control	Glycine Control	1mM Proline	10mM Proline	100mM Proline	10mM Glycine
Probes Made	126	96	72	72	72	192
Probes Counted	63	58	33	39	39	101

Table 1: Probe Count. Approximately 46% of probes made were counting and used for the statistical data.

Table 2: Fishers Test of Proline

Control to 1mM Proline			
	Probes	Cells	Totals
Control	63	304	367
1mM Proline	33	168	201
Total	96	472	568
P- Value		**0.9069**	
Control to the 10mM Proline			
	Probes	Cells	Totals
Control	63	304	367
10mM Proline	39	416	455
Total	102	720	822
P- Value		**0.0003**	
Control to 10mM Proline Excluding 103 Outlier			
	Probes	Cells	Totals
Control	63	304	367
10mM Proline w/o 103	38	313	351
Total	101	617	718
P- Value		**0.0179**	
Control to 100mM Proline			
	Probes	Cells	Totals
Control	63	304	367
100mM Proline	39	188	227
Total	102	492	594
P- Value		**1.0**	

Table 2: Fishers Test statistical analysis of varying concentrations of Proline as compared to the control. A concentration of 10mM was found to be significant as compared to the control in aggregating a cnidocyte response.

Data Collection of Glycine

Using this data, a second series of tests were performed using the optimal concentration of 10mM with the amino acid Glycine to further support the hypothesis that a) solubilized amino acids in gelatin delivered a significant effect in eliciting feeding responses as compared to a control group consisting of gelatin- coated probes and b) 10mM acted as the optimal concentration of various amino acids to enact discharge when compared to 1mM and 100mM which may deliver adverse effects. As compared to the control group, 10mM of Glycine elicited a significant positive response to feeing mechanism as shown by the amount of cnidocytes discharged and attached to the gelatin- coated probes which can be referenced in Figure 1. Statistical analysis confirmed that the probes coated with solubilized Glycine in gelatin as determined by the Fisher test (P- Value= 0.0011), Mann- Whitney Test (P- Value = 0.0), the two tailed T- Test (P- Value= .00028), and finally the Chi- square test (P- Value= .000724). A full data analysis of Glycine at a concentration of 10mM versus the control can be referenced in Table 3.

Figure 1: Boxplot of cnidocyte release of the control group, and Proline and Glycine

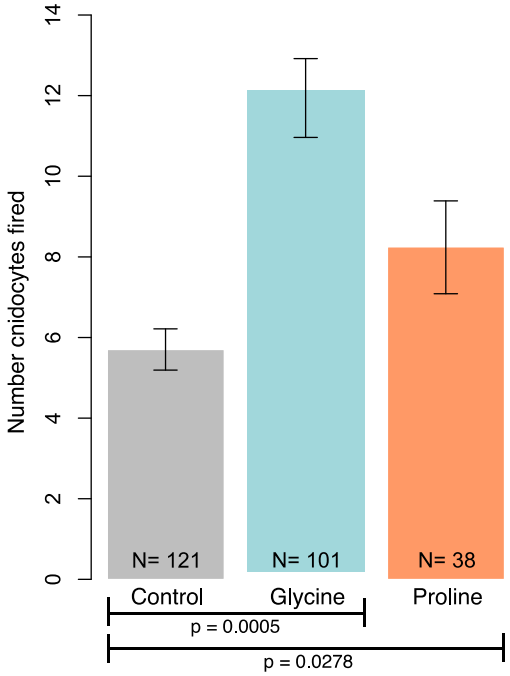

Table 3: Statistical Analysis of Glycine

Fishers Test			
Control to 10mM Glycine			
	Probes	Cells	Totals
Control	58	386	444
10mM Proline	103	1206	1307
Total	159	1592	1751
P- Value		0.0011	
	Mann- Whitney Test		
Z- Score		4.061	
P- Value		0.0	
Not/ Significant		Significant	
	Student T- Test		
T- Value		3.717	
P- Value		0.0003	
Not/Significant		Significant	
	Chi- Squared Test		
Chi- Squared		11.427	
P- Value		0.0007	
Not/Significant		Significant	

Table 3: Statistical analysis of Glycine. A concentration of 10mM was found to be significant as compared to the control in aggregating a cnidocyte response.

Statistical Comparisons

Further statistical analysis's using the Fishers Test, Mann- Whitney Test, T-Test and Chi Square Test were conducted to compare a) different concentrations of amino acids to one another b) day to day variances between the control and the Proline or Glycine experimental c) day to day variances between amino acids of the same concentrations of amino acids.

When comparing the samples of 1mM Proline to 10mM Proline and 10mM Proline to 100mM Proline, the results proved to show significance with a P- Value of 0.0043 and 0.0013 respectively while the 1mM Proline to the 100mM Proline was not found to show a significant difference in cnidocyte discharge with a P- Value of 0.90. The Mann- Whitney Test and Chi-Squared Test also showed significance with comparison of 1mM Proline to 10mM Proline with P- Values of 0.038 and 0.0030 respectively while the T- test and Chi Squared Test showed significance with 10mM Proline to 100mM Proline outputting P- Values of 0.035 and 0.0009 respectively. Results can be referenced in Table 4 for the Fisher test and Table 2 of the Appendix for supplemental data.

Table 4: Fishers Test Proline Concentrations

1mM Proline to 10mM Proline			
	Probes	Cells	Totals
1mM Proline	33	168	201
10mM Proline	39	416	455
Total	72	584	656
P- Value		**0.0043**	
1mM Proline to 10mM Proline Excluding 103 Outlier			
	Probes	Cells	Totals
1mM Proline	63	304	367
10mM Proline w/o 103	38	33	351
Total	71	48	552
P- Value		**0.0649**	
1mM Proline to 100mM Proline			
	Probes	Cells	Totals
1mM Proline	33	168	201
100mM Proline	39	188	277
Total	72	356	428
P- Value		**0.0897**	
10mM Proline to 100mM Proline			
	Probes	Cells	Totals
10mM Proline	39	416	455
100mM Proline	39	188	227
Total	78	604	682
P- Value		**0.0013**	
10mM Proline Excluding 103 Outlier to 100mM Proline			
	Probes	**Cells**	**Totals**
10mM Proline w/o 103	38	313	351
100mM Proline	39	188	227
Total	77	501	578
P- Value		**0.033**	

Table 4: Comparative statistical analysis of differing Proline concentrations using the Fisher Test. The concentration of 10mM was found to be significantly different when compared to the 1mM and 100mM concentrations while 1mM and 100mM were not significantly different to one another showing that 10mM of Proline resulted in greater cnidocyte response.

The day to day comparison was between the control and the same amino acid concentration sample as well as with the comparison between differing concentrated samples with the Fishers Test per day to show that the standardization throughout each test and date were maintained. With the exception of the first day, November 12, 2014 which had an outlier, all other days were found to be not significant for Proline indicating that the procedure and day to day testing of the *Hydra* did not adversely impact the results. Results are depicted in Table 3 of the Appendix. Glycine was conducted in a similar manner with the focus on the day to day comparisons of the control to amino acid samples and the comparison of the concentrated samples to each other. These results are seen in Table 4 of the Appendix

Discussion

Cnidocyte discharge is dictated by a complex sensory control facilitated by the opsin pathway which is hypothesized to be similar to the systems organized in vertebrates. The behavioral experiments conducted in this study demonstrated that the amino acid cues, specifically Glycine and Proline, were capable of inducing cnidocyte response. This discharge elicits a feeding response that mimics the taste one receptor responses of vertebrates to umami indicating that the feeding responses in the vertebrate systems are similar to those of invertebrates.

The optimal concentration for cnidocyte discharge was found to be at 10mM as denoted in the experiments with differing concentrations of 1mM and 100mM of Proline resulting in a P-Value of 0.003 as compared to 0.907 and 1.0 respectively. The 1mM and 100mM concentrations of Proline varied little from the discharge rates of the control possibly suggesting that those concentrations could be adversely related to the feeding mechanisms. Glycine experiments were only conducted with 10mM as a concentration and showed relative to the control, a P-Value of 0.0011 proving enhanced cnidocyte discharge as compared to the control group.

The discharge rates could have been altered on occasion due to a variety of factors including cnidocyte counting, attachment, and daily operations. The hydra were gathered each experimental day around a similar time, however, some hydra may have been exposed to different light conditions which would have induced a more phototaxis release of cnidocytes. Cnidocyte discharge is regulated by many factors including phototaxis, feeding mechanisms and locomotion. These studies did not differentiate between the different cnidocytes that were released indicating that some of the cnidocytes discharge could be attributed to causes other than

feeding responses and thus a not in relation to T1Rs. Gelatin coated probes were left to dry for approximately two days, however, depending on the thickness of the coat, drying time may have varied which would have affected the attachment of the cnidocytes to the probes.

Overall, the data shows that amino acids elicit the same feeding response in systems of vertebrates and supplemental to other data, now predates the current understanding of the evolution of T1R pathways by about 400 million years. This dating indicates that T1Rscould been the most ancient sensory receptor, present in the last common ancestor of animals and could have been present in the diversification of most animals unless lost.

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APPENDIX

Table 1: Statistical Analysis of Proline

Mann- Whitney Test				
	1mM Proline	10mM Proline	10mM Excluding 103 Proline	100mM Proline
Z- Score	-0.726	-2.827	-2.657	-1.215
P- Value	0.465	0.005	0.008	0.222
Not/Significant	Not Significant	Significant	Significant	Not Significant
Student T- Test				
	1mM Proline	10mM Proline	10mM Excluding 103 Proline	100mM Proline
T- Value	0.239	2.586	2.778	0.005
P- Value	0.811	0.011	0.007	0.005
Not/ Significant	Not Significant	Significant	Significant	Not Significant
Chi- Squared Test				
	1mM Proline	10mM Proline	10mM Excluding 103 Proline	100mM Proline
Chi- Squared	0.052	13.807	5.966	0.0
P- Value	0.820	0.0002	0.014	0.996
Not/Significant	Not Significant	Significant	Significant	Not Significant

Table 1: Statistical analysis of varying concentrations of Proline as compared to the control. A concentration of 10mM was found to be significant as compared to the control and other concentrations in aggregating a cnidocyte response.

Table 2: Statistical Analysis of Proline Concentrations

Mann- Whitney Test					
	1mM v .10mM Proline	1mM v. 10mM w/o 103 Proline	1mM v. 100mM Proline	10mM v. 100mM Proline	10mM w.o 103 v 100mM Proline
Z- Score	2.0795	1.931	0.5933	1.839	-1.676
P- Value	0.0375	0.00536	0.5552	0.0658	0.0930
Not/Significant	Significant	Significant	Not Significant	Not Significant	Not Significant
Student T- Test					
	1mM v .10mM Proline	1mM v. 10mM w/o 103 Proline	1mM v. 100mM Proline	10mM v. 100mM Proline	10mM w.o 103 v 100mM Proline
T- Value	1.8433	2.114	0.2747	2.146	2.739
P- Value	0.0696	0.0382	0.7844	0.035	0.0077
Not/ Significant	Not Significant	Significant	Not Significant	Significant	Significant
Chi- Squared Test					
	1mM v .10mM Proline	1mM v. 10mM w/o 103 Proline	1mM v. 100mM Proline	10mM v. 100mM Proline	10mM w.o 103 v 100mM Proline
Chi- Squared	8.785	5.966	5.966	11.082	4.8204
P- Value	0.003	0.05899	0.833	0.0009	0.0281
Not/Significant	Significant	Not Significant	Not Significant	Significant	Significant

Table 2: Statistical analysis of varying concentrations of Proline as compared other Proline concentrations. Tests were found to be significant for for comparison between 1mM and 10mM and 10mM and 100mM while comparisons of 1mM to 100mM were not significant indicating that the 10mM concentration of Proline elicited the greatest cnidocyte discharges as compared to other concentrations.

Statistical Analysis of Proline Daily
Fishers Test of Day to Day Comparison

11/12/2014

CONTROL TO PROLINE 10mM

	PROBES	CELLS	TOTALS
CONTROL	11	53	64
PROLINE 10mM	9	186	195
TOTAL	20	239	259
P VALUE		**0.0024**	

CONTROL TO PROLINE 10mM W/O 103

	PROBES	CELLS	TOTALS
CONTROL	11	53	64
PROLINE 10mM	8	83	91
TOTAL	19		155
P VALUE		**0.139**	

11/14/2014

CONTROL TO PROLINE 10mM

	PROBE	CELLS	TOTALS
CONTROL	12	74	86
PROLINE 10mM	11	86	97
TOTAL	23	160	183
P VALUE		**0.6584**	

11/19/2014

CONTROL TO PROLINE 100X

	PROBES	CELLS	TOTALS
CONTROL	8	32	40
PROLINE 100mM	8	28	36
TOTAL	16	60	76
P VALUE		**1**	

11/21/2014

CONTROL TO PROLINE 10mM

	PROBES	CELLS	TOTALS
CONTROL	7	35	42
PROLINE 10mM	11	82	93
TOTAL	18	117	135
P VALUE		**0.4281**	

CONTROL TO PROLINE 100mM

	PROBES	CELLS	TOTALS
CONTROL	7	35	42
PROLINE 100mM	12	74	86
TOTAL	19	109	128
P VALUE		**0.792**	

PROLINE 10 mM to PROLINE 100mM

	PROBES	CELLS	TOTALS
PROLINE 10mM	11	82	93
PROLINE 100mM	12	74	86
TOTAL	23	156	179
P VALUE	**0.8236**		

11/23/2014

CONTROL TO PROLINE 10mM

	PROBE	CELLS	TOTALS
CONTROL	10	56	66
PROLINE 10mM	8	62	70
TOTAL	18	118	136
P VALUE		**0.6159**	

CONTROL TO PROLINE 100mM

	PROBE	CELLS	TOTALS
CONTROL	10	56	66
PROLINE 100mM	9	66	71
TOTAL	19	118	137
P VALUE		**0.8057**	

PROLINE 10 mM to PROLINE 100mM

	PROBE	CELLS	TOTALS
PROLINE 10mM	9	62	71
PROLINE 100mM	8	62	70
TOTAL	17	124	141
P VALUE		**1**	

11/25/2014

CONTROL TO PROLINE 1mM

	PROBES	CELLS	TOTALS
CONTROL			
PROLINE 1mM	8	28	36
TOTAL	16	56	72
P VALUE		**1**	

CONTROL TO PROLINE 1mM

	PROBES	CELLS	TOTALS
CONTROL	8	28	36
PROLINE 1mM	8	67	75
TOTAL	16	95	111
P VALUE		**0.1477**	

CONTROL TO PROLINE 1mM

	PROBES	CELLS	TOTALS
CONTROL	8	28	36
PROLINE 1**mM**	7	40	47
TOTAL	15	68	83
P VALUE		**0.4056**	

Amino Acid to Amino Acid

11/25/2014

PROLINE 1 mM A to Proline 1mM B

	PROBES	CELLS	TOTALS
PROLINE 1mM	8	28	36
PROLINE 1mM	8	67	75
TOTAL	16	95	111
P VALUE		**0.1477**	

PROLINE 1 mM A to Proline 1mM C

	PROBE	CELLS	TOTALS
PROLINE 1mM	8	28	36
PROLINE 1mM	7	40	47
TOTAL	15	68	83
P VALUE	**0.41**		

PROLINE 1 mM B to Proline 1mM C

	PROBES	CELLS	TOTALS
PROLINE 1mM	8	67	75
PROLINE 1mM	7	40	47
TOTAL	15	107	122
P VALUE	**0.5744**		

12/3/2014

CONTROL TO PROLINE 1mM

	PROBES	CELLS	TOTALS
CONTROL	7	26	33
PROLINE 1mM	10	33	43
TOTAL	17	59	76
P VALUE		**1**	

CONTROL TO PROLINE 100mM

	PROBES	CELLS	TOTALS
CONTROL	7	26	33
PROLINE 100mM	10	24	34
TOTAL	17	50	67
P VALUE		**0.5763**	

PROLINE 1 mM to PROLINE 100mM

	PROBES	CELLS	TOTALS
PROLINE 1mM	10	33	43
PROLINE 100mM	10	24	34
TOTAL	20	57	77
P VALUE		**0.606**	

Table 3: The day to day comparison between the Control and the Proline at 1mM, 10mM, and 100mM were tested with the Fisher tests as well as analysis between the day to day analysis of the same concentration being tested on the same day as seen on 11//25/2014. With the exception, November 12, 2014, all other days were found to be not significant for Proline indicating that the procedure and day to day testing of the *Hydra* did not adversely impact the results.

Statistical Analysis of Glycine Daily Fishers Test of Day to Day Comparison												
1/20/2015				1/23/2015				1/30/2015				2/4/2015			
CONTROL TO 10mM GLYCINE A				CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE			
	PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS
CONTROL	16	179	195	CONTROL	16	91	107	CONTROL	12	48	60	CONTROL	14	68	82
GLYCINE 10mM	14	198	212	GLYCINE 10mM	9	121	130	GLYCINE 10mM	15	98	113	GLYCINE 10mM	16	120	136
TOTAL	30	377	407	TOTAL	25	212	237	TOTAL	27	146	173	TOTAL	30	188	218
P VALUE		**0.573105**		**P VALUE**		**0.056003**		**P VALUE**		**0.274722**		**P VALUE**		**0.312042**	
CONTROL TO 10mM GLYCINE B				CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE			
	PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS
CONTROL	16	179	195	CONTROL	16	91	107	CONTROL	12	48	60	CONTROL	14	68	82
GLYCINE 10mM	7	179	186	GLYCINE 10mM	15	183	198	GLYCINE 10mM	10	74	84	GLYCINE 10mM	15	233	248
TOTAL	23	358	381	TOTAL	31	274	305	TOTAL	22	122	14	TOTAL	29	301	330
P VALUE		**0.085391**		**P VALUE**		**0.048322**		**P VALUE**		**0.240547**		**P VALUE**		**0.00552**	
TOTALS				TOTALS				TOTALS				TOTALS			
CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE				CONTROL TO 10mM GLYCINE			
	PROBES	**CELLS**	**TOTALS**		**PROBES**	**CELLS**	**TOTALS**		**PROBES**	**CELLS**	**TOTALS**		**PROBES**	**CELLS**	**TOTALS**
CONTROL	**16**	**179**	**195**	CONTROL	**16**	**91**	**107**	CONTROL	**12**	**48**	**60**	CONTROL	**14**	**68**	**82**
GLYCINE 10mM	**21**	**377**	**398**	GLYCINE 10mM	**24**	**304**	**38**	GLYCINE 10mM	**25**	**172**	**197**	GLYCINE 10mM	**31**	**353**	**384**
TOTAL	**37**	**556**	**593**	TOTAL	**40**	**395**	**435**	TOTAL	**37**	**220**	**257**	TOTAL	**45**	**421**	**466**
P VALUE		**0.205072**		**P VALUE**		**0.032131**		**P VALUE**		**0.205932**		**P VALUE**		**0.021354**	
Amino Acid to Amino Acid				Amino Acid to Amino Acid				Amino Acid to Amino Acid				Amino Acid to Amino Acid			
10mM A GLYCINE to 10mM B				10mM A GLYCINE to 10mM B				10mM A GLYCINE to 10mM B				10mM A GLYCINE to 10mM B			
	PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS		PROBES	CELLS	TOTALS
GLYCINE 10mM A	14	198	212	GLYCINE 10mM A	9	121	130	GLYCINE 10mM A	15	98	113	GLYCINE 10mM A	16	120	136
GLYCINE 10mM B	7	179	186	GLYCINE 10mM B	15	183	198	GLYCINE 10mM B	10	74	84	GLYCINE 10mM B	15	233	248
TOTAL	21	377	398	TOTAL	24	304	328	TOTAL	25	172	197	TOTAL	31	353	384
P VALUE		**0.2629**		**P VALUE**		**1**		**P VALUE**		**0.8315**		**P VALUE**		**0.0761**	

Table43: The day to day comparison was between the control and the Glycine 10mM concentration Most days except 2/4/2015 show non-significant data analysis indicating that the day to day variances were minimal. Glycine to the control was found to be significant on day of 1/23/2015 and 2/4/2015.

The flavor of life: evolutionarily ancient sensory receptors for photosensitivity and taste in the cnidarian, *Hydra magnipapillata*

Connor Bell[1], Molly Hartley[1], Janine R. Appleton[1], Hannah Glanville, Julie Peterson and David Plachetzki[2]

Abstract

Animals sense their environment using specialized proteins called sensory receptors. We use the cnidarian freshwater polyp *Hydra magnipapillata* as a model to understand the early evolution of animal sensory systems. Ongoing work has identified a set of sensory genes that function in the hydra including the photosensitivity gene Opsin and Taste 1 Receptors (T1Rs). Here we report results from molecular cloning and *in situ* hybridization that demonstrates the dual expression of the photoreceptor gene Opsin and the the putatively chemoreceptive gene T1R3 in a polymodal sensory cell type in the hydra. In addition, we report results from behavioral trials that demonstrate that chemical amino acid cues from the environment are capable of exciting the discharge behavior of stinging cells called cnidocytes, mirroring the vertebrate taste sense of umami.

Background

We study the cnidarian animal *Hydra magnipapillata*, a relative of corals, jellyfish and anemones, as a model for understanding the early evolutionary histories of the animal senses

Previous work has demonstrated the importance of photosensitive opsin in mediating cnidarian feeding behavior (Plachetzki et al 2012).

Taste 1 Receptors (T1Rs) are responsible for umami and sweet taste sensation, but are presently only known from mammals (Shi and Zhang 2006)

Amino acids such as proline and glycine are capable of inducing T1R signaling, which humans perceive as savory taste

Whole genome data for representative animal genomes allow the origins of T1Rs to be explored

Results

Phylogenetic analyses of T1R genes suggest that they were present in the last common ancestor of animals and comprise one of the most ancient animal senses.

T1R genes are expressed in a polymodal sensory neuron in the hydra that also expresses photosensitive Opsins.

T1Rs may function in feeding behavior by modulating the cnidocyte (stinging cell) discharge

References

Plachetzki DC, Degnan BM, Oakley TH. The origins of novel protein interactions during animal opsin evolution. PLoS One. 2007 Oct 17;2(10):e1054. | Borowiec, M., Lee E., Chiu, J., and Plachetzki, D., 2015. Dissecting phylogenetic signal and accounting for bias in whole-genome data sets: a case study of the Metazoa. bioRxiv | Plachetzki DC, Fong CR, Oakley TH. 2012. Cnidocyte discharge is regulated by light and opsin-mediated phototransduction. BMC Biol. 2012 Mar 5;10:17. doi: 10.1186/1741-7007-10-17. | Stamatakis, A. RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. 2014. Bioinformatics btu033v1-btu033. | Shi, P. and J. Zhang. Contrasting Modes of Evolution Between Vertebrate Sweet/Umami Receptor Genes and Bitter Receptor Genes Mol. Biol. Evol. 2006 23: 292-300.

1. Whole genome analyses reveal the structure of animal phylogeny and the deep evolutionary origin of the T1R gene

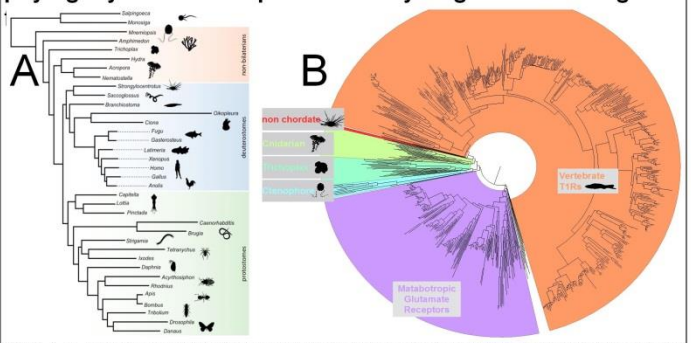

Figure 1. A. An emerging view of animal phylogeny drawn from exclusively whole genome sequences. This tree represents our current bioinformatics pipeline from which the phylogenetic history of animal T1Rs (**B**) was inferred. The animal phylogeny shown in **A** is described elsewhere (Borowiec et al 2015). For **B**, sequences of known mammalian T1R receptors were used as queries for exhaustive searches genomes depicted in **A**. Phylogenetic analyses were conducted using the best fitting model in RaXML (Stamatakis 2014).

2. Full-length T1R3 transcript cloned from the hydra using RACE

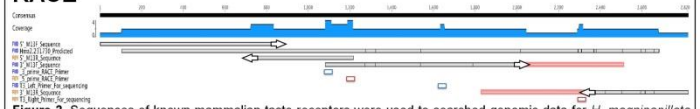

Figure 2. Sequences of known mammalian taste receptors were used to searched genomic data for *H. magnipapillata* and isolated putative T1R gene sequences. We designed four gene-specific oligonucleotide primers for use in Rapid Amplification of cDNA Ends (RACE). Two kit-specific primers were also used, which bind specifically to synthetic adaptors that were ligated to either the 5' or the 3' end of the transcript. From there, Sanger sequencing was performed on the resulting clones and the data werea ssembled using GENIOUS, resulting in the full length T1R3 clone.

3. Hydra T1R and Opsin co-localize in a polymodal neuron

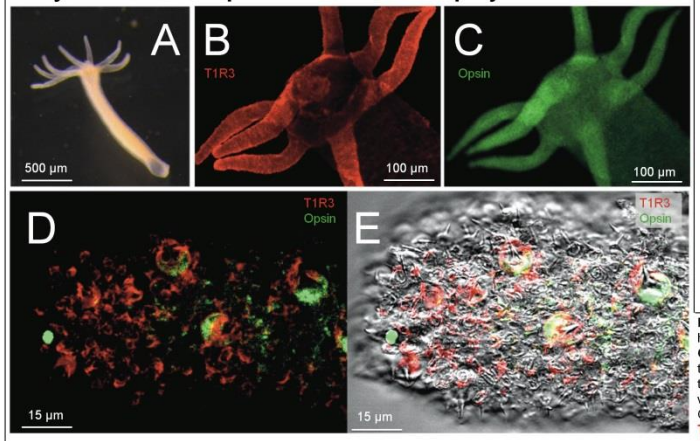

Figure 3. A. Light micrograph of living specimen of *H. magnipapillata*. **B-E.** *In situ* hybridization of sensory genes in the hydra. **B.** T1R staining (red) in whole mount. **C.** Opsin staining (green) in whole mount. **D.** T1R and Opsin co-localizae in the tentacle tip of the hydra. **E.** Confocal co-localization data merged with DIC image of tentacle tip. Riboprobes were hybridized to the transcripts of interest and the signal was developed using the Tyramide Signal Amplification system (Perklin Elmer). Confocal microscopy reveals that cnidocyte/sensory cells express both Opsin and T1R.

4. As in mammals, amino acids act as feeding cues in the hydra

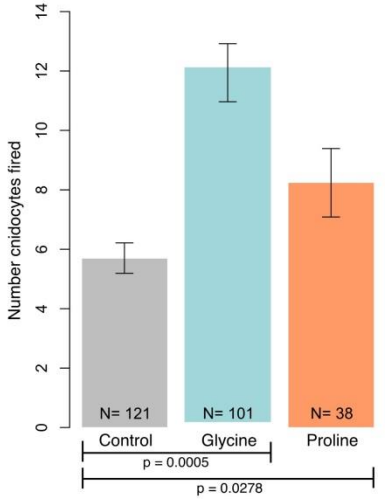

Figure 4. The effects of amino acid cues on cnidocyte activity were measured using a cnidocyte discharge assay (Plachetzki et al 2012). Discharged cnidocytes were captured using control and amino acid containing gelatin probes and analyzed using light microscopy under DIC. Cnidocyte discharge is significantly enhanced on probes containing 10mM glycine and/or proline. This experiment shows that amino acids elicit a feeding response similar in the hydra that we hypothesize is mediated by hydra T1Rs.

Conclusions

T1Rs were present in the last common ancestor of animals and function in the feeding behavior of early branching animal lineages

This finding predates the current understanding of taste receptor evolution by ~ 400 million years, indicating a possible role of T1Rs during the diversification of animals

The co-localization of chemosensitivity (taste) and photosensitivity (vision) genes in neural cells of the hydra suggests new hypotheses for the origins of animals sensory neurons

The cnidarian polymodal sensory neuron identified here is a useful model for exploring the early evolutionary history of the animal senses.