Study of Maturation of Synapses in the Human Tongue Epithelium Using Synaptophysin as Marker: An Immunohistochemical Approach

Sagnik Sen[1], Sabita Mishra[2], J. M. Kaul[3]

[1]*Intern, Lok Nayak Hospital, Maulana Azad Medical College, New Delhi, India*
[2]*Professor, Department of Anatomy, Maulana Azad Medical College, New Delhi, India*
[3]*Director Professor, Department of Anatomy, Maulana Azad Medical College, New Delhi, India*

Conflict of Interest: None declared

This article has been peer reviewed.

Article Submitted on: 1st January 2014

Article Accepted on: 17th March 2014

Funding Sources: None Declared

Correspondence to: Sagnik Sen

Address: Maulana Azad Medical College, New Delhi, India

Email: riksaq@gmail.com

Cite this article: Sen S. Study of maturation of synapses in the human tongue epithelium using synaptophysin as marker: an immunohistochemical approach. J Pioneer Med Sci. 2014; 4(3):105-111

ABSTRACT

BACKGROUND: Development of taste in higher organisms is fascinating yet poorly understood among the five senses. Many events including the development of taste receptors, neuronal connections and the gustatory cortex determine the overall advent of taste in a human fetus. This study was designed to assess the changes in the tongue epithelium of human fetuses over the age group of 14 to 20 weeks.

METHODS: Sagittal sections of tongue from 5 human fetuses of ages ranging from 14 to 20 weeks were used to study the appearance of taste buds under light microscope using hematoxylin and eosin staining, and also to observe the expression of synaptophysin, a 38 kDa integral membrane protein using immunohistochemistry with anti-synaptophysin monoclonal antibody.

RESULTS: With increasing age, an increase in the number of papillae on the dorsum of tongue was noted with maturation from primary to secondary papilla. In the 22 week fetus, probable taste buds were identified, which showed higher level of cellular organization. Faint expression of synaptophysin was seen in the epithelium and within the muscle layer in age groups of 20th and 22nd weeks, suggesting the beginning of synaptogenesis and vesicle formation in the 20 and 22 week old fetuses. From 16th week onwards, lingual glands were observed in the posterior part of the tongue, another important observation regarding taste development.

CONCLUSION: The work indicated that the maturation of taste buds and synaptic vesicles in human fetal tongue epithelium had started at around 20-22 weeks of age. This observation will pave the way for future understanding of the development of taste reception in utero.

Keywords: Taste; Human Fetus; Immunohistochemistry; Synaptophysin; Synaptogenesis; Tongue Epithelium

INTRODUCTION

Taste is one of the five senses. In humans, it is not as well developed as it is in some other mammals such as canine or feline families. Many studies have examined the evolution of taste among mammals. Chemical markers (α gustducin) have been used to identify cells called solitary chemosensory cells [1] (SCCs) in the gut of vertebrates. Fish have SCCs in oropharynx, gills and skin; amphibians have SCCs in a diffuse chemosensory epithelium; mammals have defined taste buds in the taste epithelium [1].

During taste development, it is unclear as to how the fetus is exposed to the different modalities of taste, although amniotic fluid has been proposed as conduit or source. During fetal development, the neural connections need to reach the sensory epithelium and form proper synapses with the taste receptor cells. Another unknown aspect is the approximate time during which the signal transduction due to stimulation of the receptors and movement of the stimuli begin along the neurons to the gustatory cortex. Studies on human fetal taste bud development using synaptophysin to see the neurogenesis and synaptogenesis have not been performed previously. Hence, this study was designed specifically to study the appearance of taste buds in developing human fetuses under the light

microscope using hematoxylin & eosin staining and to study the expression of synaptophysin in the pre-synaptic terminals in the tongue epithelium of developing human fetuses using immunohistochemistry with anti-synaptophysin marker antibody. Our goal was to identify developing neuron terminals and vesicle formation as this may indicate the subsequent maturation of tongue taste epithelium.

METHODS AND MATERIALS

Study duration: The study was conducted during the months of June-August, 2010.

Study place: The study was conducted in the histology laboratory of the Department of Anatomy, Maulana Azad Medical College, New Delhi, India.

Fetal Collection: For the study, 5 fetuses with ages ranging from 14 weeks to 20 weeks were obtained from the fetal repository of the Department of Anatomy, Maulana Azad Medical College, New Delhi. The fetuses were immersed in fixed 10% formalin for several weeks. Their crown rump length (CRL), crown heel length (CHL), bi-parietal diameter (BPD) and foot length (FL) were measured and according to the readings these were categorized into the following age groups (**Table 1**).

Ethical approval*:* These fetuses were cases of intra-uterine death and were collected from the labor room of Lok Nayak Hospital, New Delhi by post-graduate students in the Anatomy Department. The parents were explained about the usefulness of the dead fetuses towards academic research. They were collected only after obtaining written consent from the parents and preserved in the fetal repository of the Anatomy Department. The current study was approved by the institutional review board.

Dissection and Tissue Processing*:* Each fetus was decapitated carefully and a sagittal section was taken across the longitudinal axis of the fetus, which was further dissected to keep the tongue and palate region. The dissected specimens were taken through graded concentrations of alcohol for differential dehydration. Sagittal sections of the higher gestational age fetus had to be decalcified with 1% nitric acid before the differential dehydration for processing in paraffin. Paraffin blocks were made using L moulds after keeping the tissue sections in paraffin at temperature of 65℃. Thin sections of 5 µm were taken from each block with the help of a rotary microtome. Each section was spread in a water bath at 80^{o}C and then mounted on slides using egg albumin.

Staining with Hematoxylin and Eosin*:* Each of the ten slides of processed tissue was prepared, deparaffinised with xylene and then stained with hematoxylin and eosin.

Immunohistochemical staining using antibody against synaptophysin*:* Selected tissue sections from each fetus were deparaffinised in xylene, hydrated with decreasing concentrations of alcohol, washed with phosphate buffer (PBS), and saponified with Triton X. After blocking the activity of the endogenous peroxidases with H_2O_2 washes, tissues were incubated in 30% horse serum for nonspecific blocking. The slides were then treated with anti-synaptophysin (Serotec), a specific monoclonal primary antibody, diluted to 200 times (1:200), following which, slides were again incubated, first, in biotinylated secondary antibody (Serotec, Raleigh, NC) and second, after another wash, in streptavidin (Serotec). The tissues were treated with DAB (Diaminobenzidine; Serotec) working solution to develop peroxidase activity. The slides were then transferred to hematoxylin and distilled water. Slides were taken through increasing graded concentrations of alcohol for differential dehydration and mounted the following day. No thermal intensification was performed during staining, according to the instructions of the manufacturer. The observations were made under an Olympus BX61 computerized microscope with image proplus software and attached with the DP 71 Olympus camera for photography.

Positive control*:* Immunostained section of coch-

Table 1: Determination of ages of foetuses according to the following parameters

Foetus	CRL	CHL	BPD	FL	Age
S7	10.2	6.5	4.5	2.2	14 wks
M4	15.6	23	7	3-3.1	20 wks
S5	16.8	-	4.3	-	14-16 wks
A16	14.4	20.2	3.2	1.9-2	16-18 wks
M6	17.2	24.7	3.5	3.1-3.3	22 wks

[CRL: Crown-Rump Length; BPD: Biparietal Diameter]

lear nucleus of an 18 week fetus in a previous study was included.

Negative control: Stained section of cochlear nucleus without primary antibody.

RESULTS

In all the slides, the tip, ventral surface, dorsal surface, the anterior 2/3 and the posterior 1/3 of the tongue were identified. Slides stained with monoclonal primary antibody were analyzed separately.

14-16 weeks (S5; 10 slides): The epithelium had four layers of stratified squamous non-keratinized type. Below the epithelium, many vacuolated spaces were seen. The tip and dorsal surface were characterized by developing papillae. The ventral surface was lined with stratified squamous epithelium without any papillae (**Ages 14-16 weeks Fig 1A.**)
The bulk of the tongue had developing skeletal muscle cells and other cells of different morphology. Numerous red blood cells (RBCs) were seen distributed in the muscle layer (**Ages 14-16 weeks Fig. 1B.**). Few cells had processes; these were probably the migratory or wandering cells, which resemble the typical neural crest cells, moving towards the epithelium.
Just below the epithelium, whorls were observed, which might represent the developing taste papillae, as they appeared to be surrounded by elongated cells with round nuclei, which may be the basal cells of the epithelium.
At the base of the developing papillae, there were multiple cell types- cells with elongated nuclei, those with round nuclei and those with flattened nuclei. Many of these nuclei were dark stained and some others were light stained. Also present were cells with euchromatic nuclei, representing mitotic cells. No expression of synaptophysin could be seen.

16-18 weeks (A16; 10 slides): The epithelium was six layered stratified squamous non-keratinized type. The tip and dorsum were characterized by the abundance of thin developing filiform papillae and few fungiform papillae developing on the lateral lingual sides while circumvallate papillae were anterior to the foramen caecum (**Ages 16-18 weeks Fig. 2A.**). The vacuolated spaces were much smaller and mesodermal proliferation was seen. The muscles were forming the bulk of the tongue. The ventral surface demonstrated abundance of mucous

Figure 1 (A) Age 14-16 weeks: This depicts the epithelium and the vacuolated spaces beneath the epithelium. The papillae have just started developing (10x)

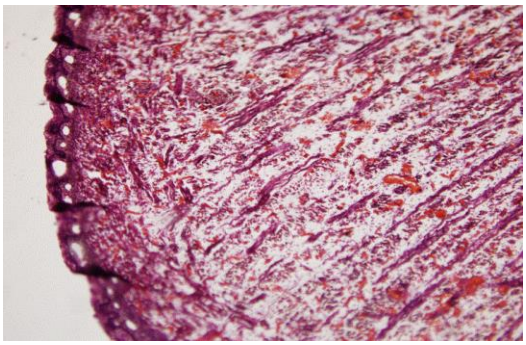
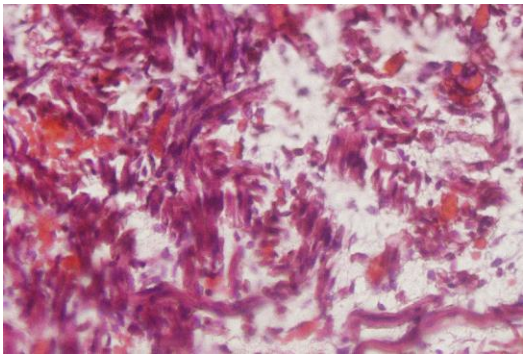


Figure 1 (B) Age 14-16 weeks: Developing muscle fibers. Many of them cannot be called true muscle fibers but are on the of process development. Numerous RBCs are seen distributed in the muscle layer (40x)



acini, the developing lingual glands. The epithelial whorls were qualitatively more in number in comparison to the previous age, and the cells were more organized. It appeared that the whorls increased in dimensions. Cells in whorls were more compactly arranged with prominent mitotically active cells with euchromatic nuclei. Central region of whorls was vacuolated and a few cells were present here, which may be the neural cells. No expression of synaptophysin could be seen.

20 weeks (M4; 10 slides): The epithelium was seven layered stratified squamous non-keratinized type. Papillae were identified. The developing skeletal muscles were forming the bulk of the tongue. The ventral surface demonstrated abundance of mucus acini (developing lingual glands). Further differentiation of whorls was seen. The whorls had moved further towards the epithelium. 2-3 triangular neuronal cells with thin, light cytopla-

Figure 2 (A) Age 16-18 weeks: The fora -men caecum is visible. The remnant of the thyroglossal duct is seen. Many whorls (Wh) are visible. The circumvallate Papillae (CVP) are present anterior to the foramen caecum. Cut sections of ducts of lingual glands are seen below the epithelium (10x)

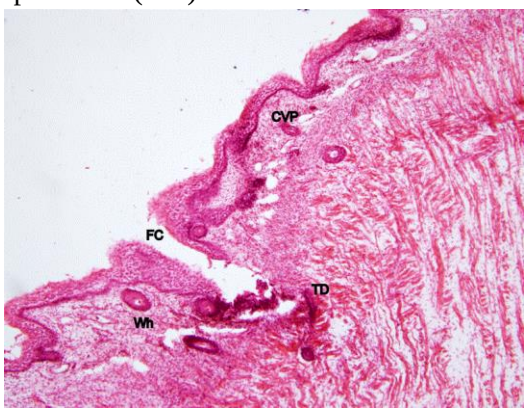
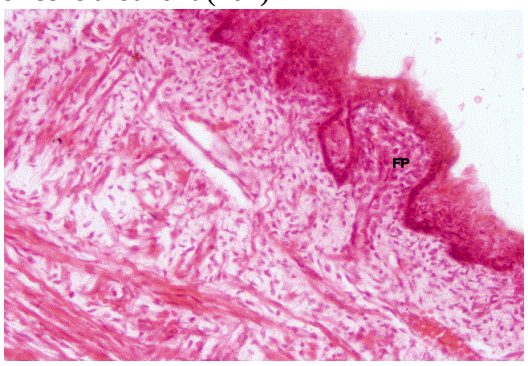


Figure 2 (B) Age 16-18 weeks: A Fungi-Form Papilla (FP) is seen under high magnification, showing the organization of cells around it (40x)



sm and round nuclei were observed, which were surrounded by cells with elongated nuclei and these may be supporting cells. Faint expression of synaptophysin was seen (**Ages 20 weeks Fig. 2B.**).

22 *weeks (M6; 10 slides):* The epithelium was nine layered stratified squamous non-keratinized type. Papillae were identified. The muscles were forming the bulk of the tongue. The ventral surface demonstrated abundance of the developing lingual glands. Probable taste buds were seen along the lateral and basal walls of the fungiform papillae and lateral walls of circumvallate papillae. In the developing taste buds, cells with elongated nuclei were observed, which might be the basal cells, and cells with round nuclei, which might be sustentacular cells differentiating from basal cells. Also a cell with a process was observed, which was identified as a neural cell. Organization of cells in the whorls continued. No adult arrangement of cells seen under light microscope. Faint expression of synaptophysin was seen at 22 weeks in the sub-epithelial part of the dorsum of the tongue **(Ages 22 weeks Fig 4C.).**

DISCUSSION

Perception of the sense of taste in humans is present in the tongue and the palate region. Tongue has taste buds on the fungiform, foliate and vallate (circumvallate) papillae and the palate has taste buds in the nasoincisor papilla/duct and on the soft palate. Filiform papillae do not have taste buds. The circumvallate and the filiform papillae are located in the central and lateral parts

Figure 3 (A) Age 20 weeks: Lingual glands (LG) developing (mucus acini)(10x)

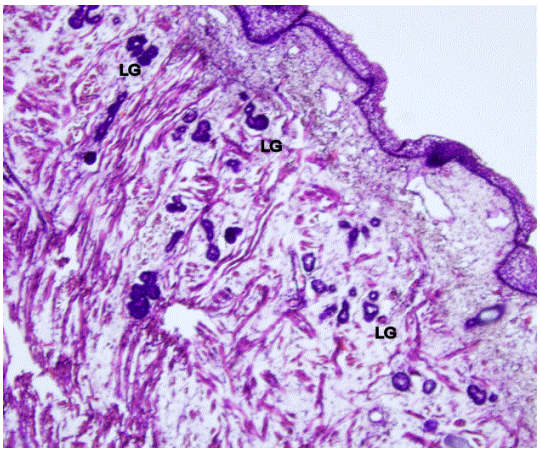


Figure 3 (B) Age 20 weeks: Faint expression of Synaptophysin recorded (10x)

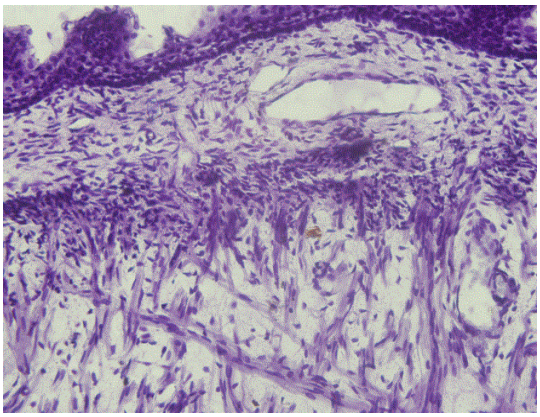


Figure 4 (A) Age 22 weeks: Probable taste buds have been pointed. The figure shows a papilla in a tangential section showing the mesodermal core (40x)

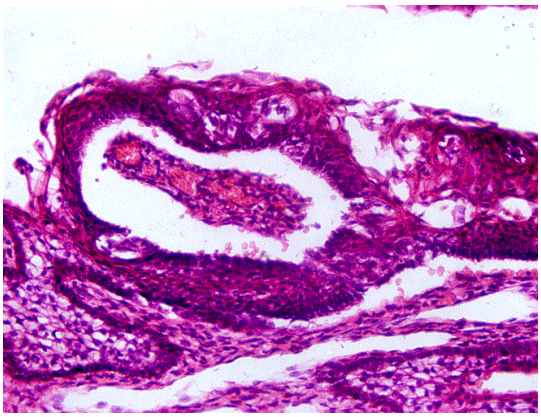


Figure 4 (B) Age 22 weeks: The developing taste bud has been magnified under oil immersion. Organization of cells is seen taking place in it. A neuropore is also seen in the taste bud (100x).

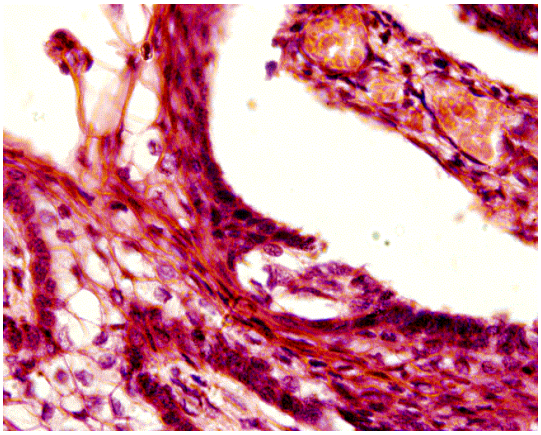
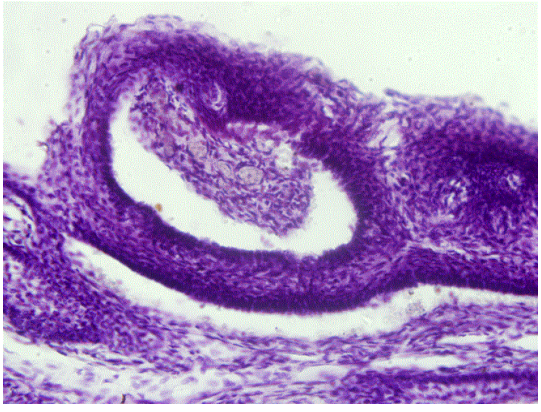


Figure 4 (C) Age 22 weeks: Faint expressi -on of Synaptophysin is seen in the papilla (40x)



of the tongue and the fungiform papillae are in the anterior most part of the tongue [4].

In the present study, fetuses from the age of 14 weeks to 22 weeks were taken to study the development of papillae, organization of taste buds and the synaptogenesis in the tongue epithelium. The epithelium with multiple layers was identified, which was characterized by papillae in the dorsum and tip of the tongue. The number of layers in the stratified squamous epithelium increased from four to nine from 16th to 22nd week. In 14-16 weeks, the papillae were developing and vacuolated regions were present below the epithelium. In the vacuolated spaces, muscle cells were present and some muscle cells were migrating into the spaces. All throughout the muscle layer and in the mesoderm below the epithelium, numerous RBCs were seen indicating immature vascularization. The taste papillae develop from epibranchial placodes [5] which are formed due to mesenchymal proliferation, and the taste neuronal fibers are also guided towards these regions by brain-derived neurotrophic factor (BDNF) expressed by the epithelial cells [6][7][8]. Recent studies have suggested that mammalian and axolotl taste buds may also be induced independently of innervation [5]. The lingual (submandibular) ganglia and the papillae on the tongue initially develop independently [9], but then become interconnected. The ganglia derived neurotrophic factors produced by the gustatory papillae induce the development of sensory innervation from the ganglia, which in turn helps papillae growth and morphogenesis. Whorls were seen developing from 16th week onwards and they were seen to increase in dimension and volume. In the age group of 22 weeks, blood vessels were seen in the whorls, which suggested that these were the mesodermal core of the developing papillae. Their appearance suggested that they were cut tangentially during tissue sectioning. Across the ages, there was an increase in the number of papillae on the dorsum of tongue. In 16-18 weeks, primary papillae were visible which changed to secondary papillae in the later ages of 20 and 22 weeks. Thus, it can be said that the papillae are maturing throughout 14 to 22 weeks. In the 22 week fetus, probable taste buds were identified, which showed organization of cells into those with rounded nuclei, the probable basal cells, those with elongated nuclei, the probable sustentacular cells and an elongated cell with a process, a typical neural cell. Taste buds consist of modified epithelial cells [7], and they contain taste pores, into which project microvilli of taste receptor cells (TRCs) [10].

Taste receptor cells, despite being epithelial cells, resemble neuronal cells [7]. It is now known that neural crest cells contribute to the formation of the taste buds. Studies have found that the taste bud development gets completed by the 15th week and by the 12th week; many differentiated new cells were seen [11]. The observations were different from the literature, as the probable taste buds were found to be appearing in 22nd week fetus and not in 14th to 20th week fetuses.

In the probable taste bud, taste pore was observed, which was identified as a neuropore. According to literature [11], by the 14th-15th week, taste pores have developed, which again is different from the study observation, which showed no presence of taste pores before 22nd week. Literature says that till the appearance of mucus in the taste pores, it can be said that the taste bud is still not fully developed. Taste pore is the last component of a taste bud to develop [11] and do so with the help of undifferentiated marginal cells.

Von Ebner's glands or the posterior lingual glands, located beneath the circumvallate papillae [14], release their secretion (mostly amylases) in the lower part of the grooves between the circumvallate papillae so that the TRCs are present in an environment of amylases released from these glands. The glands help in gustatory sensation by providing the environment for reception and helping in trapping of the tastants in the environment. From 16th week onwards, development of lingual glands was observed in the posterior part of tongue. This is significant with taste reception [14].

Synaptophysin, a Ca^{2+}-binding component of the vesicle membrane, is a 38 kDa integral membrane protein [3] with four transmembrane domains and a cytosolic C-terminal residue. Synaptophysin marker antibody binds to the C-terminal residue and indicates the site where tongue epithelium vesicle formation takes place. For studying the beginning of synaptogenesis in the tongue epithelium, a marker specific for synaptophysin was used, which should be present in the presynaptic ends of the terminal branches of the gustatory neurons and the bases of the taste receptor cells. In previous studies, synaptophysin has been found in small, round or flat synaptic vesicles but absent from large dense-cored vesicles [12]. Thus, it is an indicator of vesicle formation. Moreover, synaptophysin is used as a marker of neurogenesis and terminal neuronal differentiation. In several immunohistochemical studies with synaptophysin, thermal intensification before labeling with primary antibody has provided better results by increasing the immunoreactivity due to better antigen retrieval [13]. However, no thermal intensification was performed during staining for this study, according to the recommendations written by the manufacturer.

In the present study, no expression of synaptophysin was detected in the age groups of 14^{th} to 18^{th} weeks. However, there was faint expression of synaptophysin below the epithelium and within the muscle layer in the ages of 20 and 22 weeks. This suggested that synaptogenesis and vesicle formation had begun in the age groups of 20 and 22 weeks. This expression of the protein in the muscle layer pointed to its anterograde transport from the ganglionic cell bodies to the axonal endings of sensory neurons, where it probably participates in the regulation/modulation of neurotransmitter release by the taste receptor cells. By comparing the immunohistochemically stained fetal tissues against the adult tongue, trends in the amount of synaptogenesis during development from fetus through post-natal stage to adult can be studied.

A limitation of this study is a relatively small number of fetuses included which may limit generalization. Comments on the exact duration of development of the papillae cannot be made solely on the basis of a light microscopic study and moreover, the observations cannot be compared with electron-microscopic findings of past studies. It has not been possible to differentiate among the different cell lineages, as differential staining was not performed.

CONCLUSION

In conclusion, we show that differentiation of papillae start between 14 and 16 weeks and secondary papillae were present by 20 weeks. Taste buds organization started around 22 weeks with faint expression of synaptophysin; therefore, maturation of the taste buds takes place after 22 weeks of gestation.

ACKNOWLEGEMENTS

I am extremely grateful to Dr. J. M. Kaul and Dr. Sabita Mishra from the Anatomy Department, Maulana Azad Medical College, my teachers, without whose kind guidance, this study would not have been possible.

REFERENCES

1. Sbarbati A, Osculati F. Solitary Chemosensory Cells in

Mammals. *Cells Tissues Organs*. 2003; 175:51-5
1. Hilbe M, Guscetti F, Wunderlin S, et al. Synaptophysin: an Immunohistochemical Marker for Animal Dysautonomias. *J Comp Path*. 2003; 132: 2-3
2. Jahn R, Scheibler W, Ouimet C, Greengard P. A 38000-Dalton membrane protein (p38) present in synaptic vesicles. *Proc Natl Acad Sci*. 1985; 82:4137-4141.
3. Jung HS, Akita K, Kim JY. Spacing patterns on tongue surface-gustatory papilla. *Int J Dev Biol*. 2004; 48: 157-161
4. Shoba T, Danielle E. Harlow, Amanda L et al. Barlow Fate mapping of mammalian embryonic taste bud progenitor. *Dev J*. 2009; 136: 1519–1528
5. Patel AV, Krimm RF. Role of brain-derived neurotrophic factor in target invasion in the gustatory system. *J Neurosci.* 1999; 19:3507-3518.
6. Gray H. Gray's Anatomy. Elsevier. 39 edition.
7. RF Krimm. Factors that regulate embryonic gustatory development. *BMC Neurosci*. 2007; 8 Suppl 3:S4.
8. Ito A, Nosrat IV, Nosrat CA Taste cell formation does not require gustatory and somatosensory innervation. *Neurosci Lett*. 2010; 471:189-194.
9. Chandrashekar J, Hoon MA, Ryba NJ, Zuker CS. The receptors and cells for mammalian taste. *Nature.* 2006; 444:288-294.
10. Witt M, Reutter K. Scanning electron microscopical studies of developing gustatory papillae in humans *Chem Senses*. 1997; 22:601-612
11. Navone F, Greengard P, DeCamili P. Synapsin I in nerve terminals: selective association with small synaptic vesicles. *Science.* 1984; 226:1209.
12. Harvey B, Donald E. Synaptophysin immunocytochemistry with thermal intensification: a marker of terminal axonal maturation in the human fetal nervous system. *Brain Dev*. 1999; 21:41-50.
13. Schmale H, Holtgreve-Grez H, Christiansen H. Possible role for salivary gland protein in taste reception indicated by homology to lipophilic-ligand carrier proteins. *Nature.* 1990; 343: 366-369