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Original article

Expression of N-cadherin and cell surface molecules in the taste buds of mouse circumvallate papillae

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ABSTRACT

Objectives: Cadherins are a type of adhesion molecule involved in cell–cell recognition and morphogenesis. N-cadherin is predominantly expressed in the nervous system, and is localized at synapses, where it not only plays an adhesive role, but also participates in the regulation of synaptic function and plasticity. Taste cells within taste buds have a limited lifespan, and are replaced on a regular basis. However, little is known regarding the expression pattern of cadherins in taste bud cells. In this study, we examined whether taste bud cells in mouse taste papillae express N-cadherins, and if so, which cell type (s) the N-cadherins are found in.

Methods: We examined the expression of N-cadherins by reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, and *in situ* hybridization.

Results: RT-PCR results demonstrated that N-cadherins are expressed in the taste bud-containing epithelium of the circumvallate papilla in mice. Based on immunohistochemical analyses, N-cadherins were found to be expressed in a subset of taste bud cells of gustatory papillae. Double-labelling studies showed that N-cadherin co-localizes with α-gustducin, aromatic L-amino acid decarboxylase (AADC), carbonic anhydrase IV (CA4), and phospholipase C β2 (PLCβ2).

Conclusions: Our study indicated that N-cadherins are expressed in type II and III taste cells. Taken together with results from previous studies, we propose that N-cadherins might play a functional role in the establishment of nerve terminal connections.

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1. Introduction

Cadherins are essential for maintaining multicellular structures, and play a role in vital processes such as embryogenesis, pattern formation, and maintenance of specific tissue architectures. Classic cadherins are Ca^{2+}-dependent adhesion molecules that contain five extracellular subdomains, which are separated from the cytoplasmic domain by a single transmembrane segment [1]. The cytoplasmic domain binds to and interacts with catenins, which transduce signals and serve as bridges to the cytoskeleton [2]. During development, cadherins assist in proper positioning of cells and morphogenesis [3,4]; in nervous tissues, N-cadherin acts as the target for nerve terminals [5,6] and in the formation of synapses [7,8]. N-cadherin, a member of the cadherin superfamily of cell adhesion molecules, mediates Ca^{2+}-sensitive homophilic binding between apposed cell membranes, and is implicated in neurite outgrowth, dendritic arborization, axon guidance, and early stages of synaptogenesis [9,10].

Taste buds are chemoreceptors that function as end-organs for taste. In mammals, most taste buds are located in the stratified squamous epithelium of the dorsal surface of the tongue, and are concentrated in circumvallate, foliate, and fungiform papillae. Gustatory cells found in taste buds have been identified as paraneurons, as they possess characteristics of both neuronal and epithelial cells [11]. Like neurons, these cells form synapses, store and release transmitters, and are capable of generating action potentials [12,13]. Like epithelial cells, mammalian taste cells have a limited lifespan, and are regularly replaced from approximately 10 proliferative basal stem cells per bud [14–17]. Therefore, gustatory nerve terminals need to establish new connections on a regular basis with newly formed cells. However, little is known regarding the mechanisms that allow gustatory nerve fibers to locate appropriate taste cells.

In this study, we examined whether taste bud cells in mouse

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taste papillae express N-cadherins, and if so, in which cell type(s) the N-cadherins are located. We performed double immunostaining for N-cadherin and aromatic L-amino acid decarboxylase (AADC), carbonic anhydrase 4(CA4), α-gustducin, or phospholipase C β2 (PLCβ2). We further examined the expression pattern of N-cadherin mRNA in mouse taste buds.

2. Materials and methods

2.1. Animals

Adult ICR mice (10-weeks-old) of both sexes (male: 4, female: 3) were used for this study. Use of these animals was approved by the Kyushu Dental University Animal Care and Use Committee, and all animal protocols conformed to the National Institute of Health guidelines.

2.2. Tissue preparation

Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and were perfused through the left ventricle with 4% paraformaldehyde (PFA) in phosphate buffer (pH 7.4). Tongues of perfused mice were fixed overnight in the same fixative, and were embedded in OCT compound (Sakura, Torrance, CA, USA). Cryostat Section (4–6 μm) were mounted on MAS-coated Superfrost slides (Matsunami Glass Ind., Osaka, Japan), and were stored in airtight boxes at −80 °C.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR, dissected tongue tissues containing circumvallate papillae, as well as tongue tissues from non-gustatory regions, were incubated for 60 min at 37 °C with 2% type IV collagenase (Sigma) in supplemented a-MEM (Cosmo Bio. Co., Ltd.). Following the incubation period, the papillary epithelium was manually separated from the underlying connective tissues using fine forceps. Total RNA was isolated from the epithelium of circumvallate papillae, the brain, and tongue epithelium without taste buds; isolated RNA was incubated with DNase I, and was reverse-transcribed using oligo-dT primers and avian myeloblastosis virus (AMV) reverse transcriptase at 42 °C for 4 h to yield single-stranded cDNAs. Following denaturation at 94 °C for 120 s, PCR amplification was performed under the following conditions: 94 °C for 30 s, 53 °C for 30 s, and 72 °C for 1 min for a total of 35 cycles. The reaction was terminated after a 15-min elongation step at 72 °C. The reverse transcriptase step was omitted in negative control samples to confirm the removal of all genomic DNA. Amplification products were analyzed on 2% agarose gels, and were visualized with ethidium bromide. Amplification products were sub-cloned and sequenced for confirmation of identities. The sequences of the primers used were as follows:

N-cadherin: 5′-AGGGTGGACGTCATTGTAGC-3′ (forward) and 5′-CGGTTGATGGTCCAGTTTCT-3′ (reverse); β-actin: 5′-CACCCTGTGCTGCTCACC-3′ (forward) and 5′-GCACGATTTCCCTCTCAG-3′ (reverse).

2.4. In situ hybridization

Tissue sections were processed as previously described [18]. In brief, rehydrated sections were treated for 10 min with 0.2 N HCl, followed by 5 min incubation with proteinase K (1 μg/ml in Tris-ethylenediaminetetraacetic acid (EDTA)). Sections were then washed in phosphate buffered saline (PBS), re-fixed for 20 min in 4% PFA, and treated twice for 15 min with glycine (2 mg/mL in PBS). Sections were pre-hybridized for 1 h at room temperature in hybridization buffer. Digoxigenin-labeled antisense and sense riboprobes were produced from plasmids containing N-cadherins. Hybridization was performed overnight at 60 °C in hybridization buffer containing 0.5–1.0 μg/ml riboprobe. Excess probe was removed by sequential washes, and sections were blocked for 1 h in 1% blocking reagent-containing maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl). Tissue sections were then incubated for 2 h with anti-digoxigenin antibody conjugated to alkaline phosphatase (diluted 1:250 in blocking solution). Antibody binding was visualized with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP). Prior to imaging or immunohistochemistry, sections were re-fixed in 4% PFA.

2.5. Immunohistochemistry

For immunohistochemical staining, tissue sections were blocked for 2 h in 5% goat serum in PBS, and were incubated with primary rabbit anti-N-cadherin (1:400; Novus Biologicals, NB600-1038, Lot No. 006FDF) overnight at 4 °C in a humidified chamber. Following a PBS rinse, sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) overnight at 4 °C. Slides were again rinsed with PBS, and were coverslipped with Vectashield (Vector Laboratories, USA). The specificity of N-cadherin immunoreactivity against mouse tissues was determined by substituting a buffer for the primary antibody.

For double immunohistochemical staining, tissue sections were incubated with anti-N-cadherin (1:400) and anti-carbonic anhydrase IV (CA4) (1:200; R&D system) primary antibodies. Alexa Fluor 488 conjugated donkey anti-rabbit IgG (1:1000) and Alexa Fluor 546 conjugated donkey anti-goat IgG (1:1000) were used as secondary antibodies.

For double immunohistochemical staining using primary antibodies from the same host species, the following procedure was used [19]: sections were first incubated with anti-N-cadherin (1:400) primary antibody and with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000) secondary antibody. After rinsing with PBS, sections were sequentially incubated with anti-rabbit Fab fragment (1:100; Jackson laboratory), anti-gustducin (1:1000; Santa Cruz), anti-PLCβ2 (1:1000; Santa Cruz), or anti-AADC (1:200; GeneTex) primary antibodies overnight at 4 °C. Alexa Fluor 568 conjugated-goat anti-rabbit IgG (1:1000) was used as the secondary antibody. Slides were rinsed with PBS, and were coverslipped with Vectashield (Vector, USA). Negative controls were generated by omitting primary antibodies.

All images were obtained by changing the filter cube on a cooled CCD camera (Olympus); no alteration was made to the focus and the x/y coordinates of the section. The contrast and color of the digital images were adjusted, and figure plates were created using the Adobe Photoshop CS5 program for Macintosh.

2.6. Data analysis

For quantification, alternate section was examined to avoid counting the same cells. Immunopositive cells were defined by the presence of nuclear profile.

3. Results

3.1. RT-PCR analysis

We performed RT-PCRs to determine N-cadherin gene expression in mouse taste buds (Fig. 1). RT-PCR was performed using RNAs prepared from brain tissues, circumvallate papilla epithelium, and areas of the tongue epithelium that did not contain taste buds. The expected amplicon size with primer sets specific for mouse N-cadherin was 586 bp; amplified cDNAs were sequenced

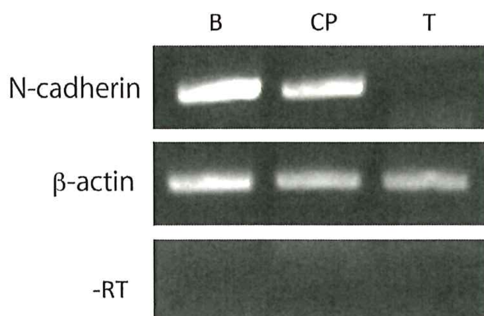

Fig. 1. Analysis of N-cadherin expression in mouse tongue. RT-PCR was performed using total RNAs isolated from the brain (B), the epithelium of circumvallate papillae (CP), and tongue epithelium that does not contain taste buds. β-actin was used as control; the reverse transcriptase step was omitted in negative controls. The expected size for amplicons obtained with primer sets specific for mouse N-cadherin is 586 bp.

to confirm identity. As expected, RNAs from tongue epithelium lacking taste buds did not yield amplification products.

3.2. Expression of N-cadherin in taste buds

We used *in situ* hybridization to examine the expression pattern of N-cadherin mRNAs in mouse taste buds of circumvallate papilla. N-cadherin mRNAs were found in taste buds of adult mouse circumvallate papilla (Fig. 2A). N-cadherin-expressing cells were detected in taste buds, but were not observed in other papillary epithelial elements. Within the taste buds, N-cadherin-expressing cells were found to be localized in small numbers in fusiform cells.

Sections of circumvallate papillae from adult mice were also immune-reacted with anti-N-cadherin antiserum. N-cadherin was expressed in a large subset of taste bud cells and nerve terminals in circumvallate papillae (Figs. 3 and 4 left panel). However, N-cadherin expression was not observed in other papillary epithelial elements, such as epithelium between taste buds (Figs. 3 and 4 left panel). In taste buds, anti- N-cadherin antibodies reacted with cell membranes of slender, spindle-shaped cells that extended across the entire height of the membrane (Figs. 3 and 4 left panel). One to five immune-positive cells were present in each taste bud section. Negative controls showed no specific labeling (data not shown).

3.3. Double staining for N-cadherin and taste cell markers

To further assess which cell types express N-cadherins in taste buds, we double-labelled cells with N-cadherin and other taste cell marker antibodies. α-gustducin is a marker of a subset of type II taste cells; α-gustducin immunoreactive taste cells were pyriform-shaped with large round nuclei (Fig. 3B). Approximately 37% of N-cadherin immunoreactive cells showed α-gustducin immunoreactivity (36.5%, 167/457; Fig. 3A–C, Fig. 5). PLCβ2 is a marker of type II taste cells, and is also present in a subset of type III taste cells [20]. PLCβ2 was present in a large number of mouse circumvallate taste buds (Fig. 3E). In addition, co-localization of N-cadherin and PLCβ2 were observed in many cells (54.1%, 244/451; Fig. 3D–F arrows, Fig. 5). AADC and CA4 are markers of type III taste cells. Slender, spindle-shaped cells were immuno-reactive for AADC and CA4 (Fig. 4B, E). Approximately 40% of taste cells that displayed N-cadherin immunoreactivity also demonstrated immuno-reactivity for AADC and CA4 (AADC, 39.3%, 141/359; CA4, 41.2%, 196/476; Fig. 4A–F arrows, Fig. 5); however, some N-cadherin positive cells were negative for type III cell markers (Fig. 4A–F closed arrowheads).

4. Discussion

In the present study, we have demonstrated for the first time that N-cadherin is expressed in mouse taste buds. Using RT-PCR, the presence of N-cadherin mRNA was confirmed in the epithelia of circumvallate papilla, which contain taste buds. An antiserum against N-cadherin also demonstrated positive signal in a large subset of taste bud cells in circumvallate papillae.

Based on the ultrastructural features of taste bud cells, mammalian taste buds are classified into four distinct morphologically identifiable types of cells [21]. In this classification of taste cells, type II and type III cells have been designated as taste receptor cells, whereas type I cells have been proposed to play a supportive or glia-like role [22,23]. Previous studies have shown that type II cells are marked by α-gustducin -immunoreactivity (IR), and PGP9.5-IR [24–26]. In contrast, the most distinct features of type III cells are the presence of numerous vesicles and their synaptic contact with nerve terminals [21]. Subsets of type III cells are either AADC-IR or CA4-IR [27,28]. In addition, PLCβ2 is a marker of type II taste cells, and is also present in a subset of type III taste cells [20]. In this study, N-cadherin-expressing taste cells demonstrated α-gustducin, AADC, CA4, and PLCβ2 expression. These results suggested that N-cadherin-expressing taste cells are type II and type III cells.

Cadherins is one of the adhesion molecules involved in cell–cell recognition and morphogenesis [29]. It has been shown that most members of the classic cadherin family preferentially mediate homophilic interactions between cells, which induces cell segregation based on the type of cadherin they express [3]. N-cadherin is predominantly expressed in the nervous system, and localizes at synapses [30]. It not only plays an adhesive role, but also participates in the regulation of synaptic plasticity [31,32]. The sensation of taste includes five established basic tastes: bitter, salty, sour, sweet, and umami. Recent studies have determined candidate receptors for the five basic tastes [33,34]. The expression patterns of these receptors suggest that separate populations of taste bud cells are responsible for each taste quality [35]. Taste cells have a limited lifespan, and are

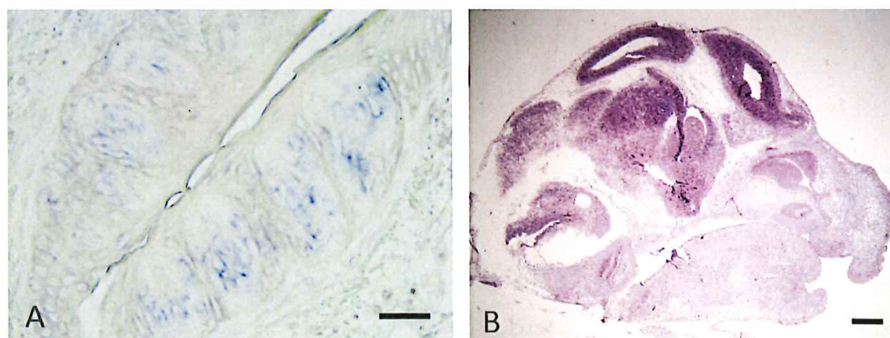

Fig. 2. N-cadherin *in situ* hybridization. *In situ* hybridization for N-cadherin in mouse circumvallate papilla (A) and embryo head (E14) (B) was performed. Embryo Head (E14) was used as a control. Scale bars = 15 μm in (A), 200 μm in (B).

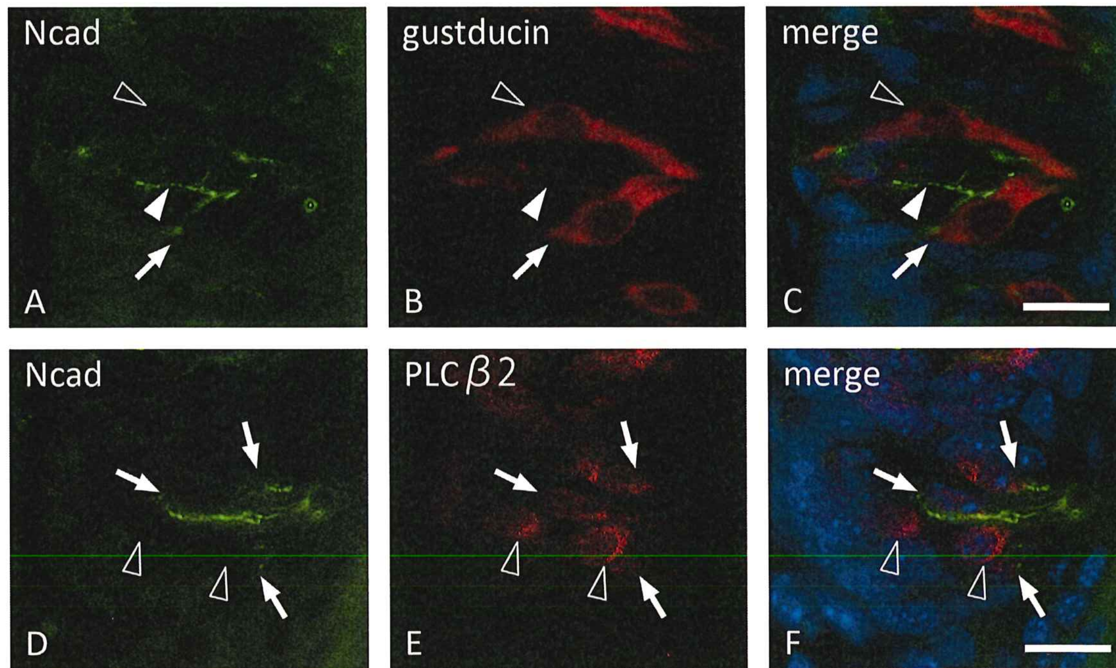

Fig. 3. Double-labeled immunohistochemical images of N-cadherin and α-gustducin or PLCβ2 in mouse circumvallate taste buds. A–C: Immunofluorescence of N-cadherin (green, A), α-gustducin (red, B), and their overlay (C) in longitudinal sections; taste cells immuno-reactive for both α-gustducin and N-cadherin immunoreactivity are indicated by arrows. D–F: Immunofluorescence of N-cadherin (green, D), PLCβ2 (red, E), and their overlay (F) in longitudinal sections; cells immuno-reactive for both PLCβ2 and N-cadherin are indicated by arrows. Closed arrowheads indicate N-cadherin immuno-positive cells that lack α-gustducin or PLCβ2 staining (A–F). Open arrowheads indicate cells positive for only α-gustducin or PLCβ2 (A–F). Scale bars = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

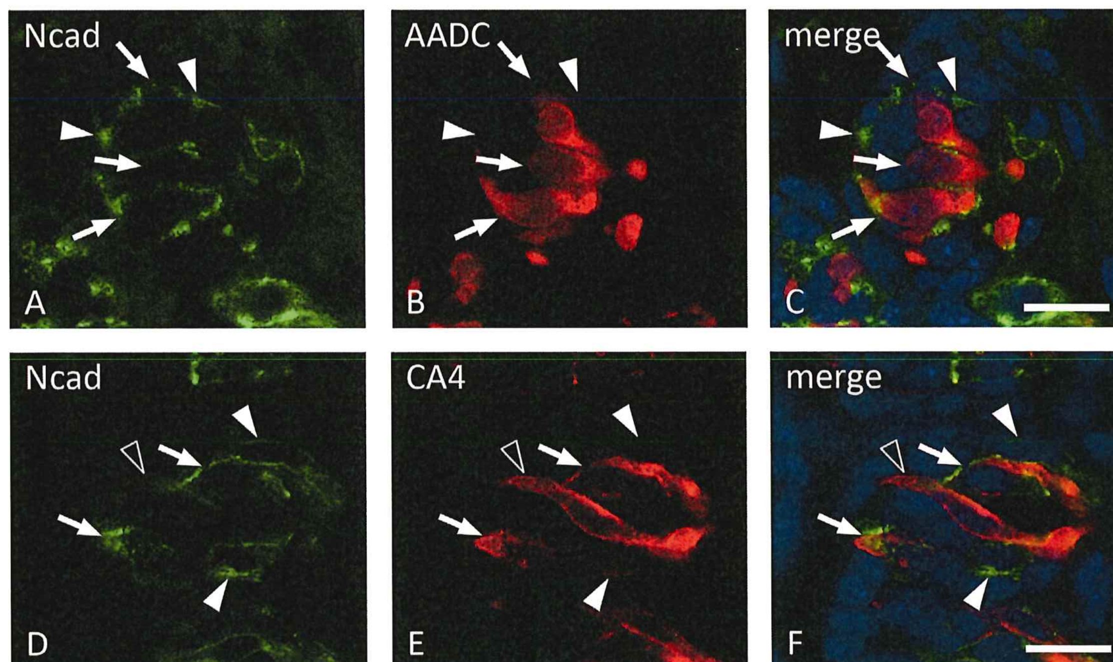

Fig. 4. Double-labelled immunohistochemical images of N-cadherin and AADC or CA4 in mouse circumvallate taste buds. A–C: Immunofluorescence of N-cadherin (green, A), AADC (red, B), and their overlay (C) in longitudinal sections. Taste cells immuno-reactive for both AADC and N-cadherin are indicated by arrows; taste bud cells exhibiting only N-cadherin-immunoreactivity are illustrated by closed arrowheads. D–F: Immunofluorescence of N-cadherin (green, D), CA4 (red, E), and their overlay (F) in longitudinal sections. Taste cells immuno-reactive for both CA4 and N-cadherin are indicated by arrows; taste bud cells exhibiting only N-cadherin-immunoreactivity are indicated by closed arrowheads; taste bud cells exhibiting only CA4-immunoreactivity are indicated by open arrowheads. Scale bars = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regularly replaced within the taste buds. In addition, nerve terminals that transduce taste signals from taste cells need to re-establish connection with replaced taste cells that express the same type of taste receptors [36]. This suggests that a mechanism is in place to facilitate the correct selection of taste receptor cells in order to establish accurate nerve terminal connections. Takeichi previously reported that a combination of different cadherins could create a wider variety of adhesive specificities between cells [1]. We

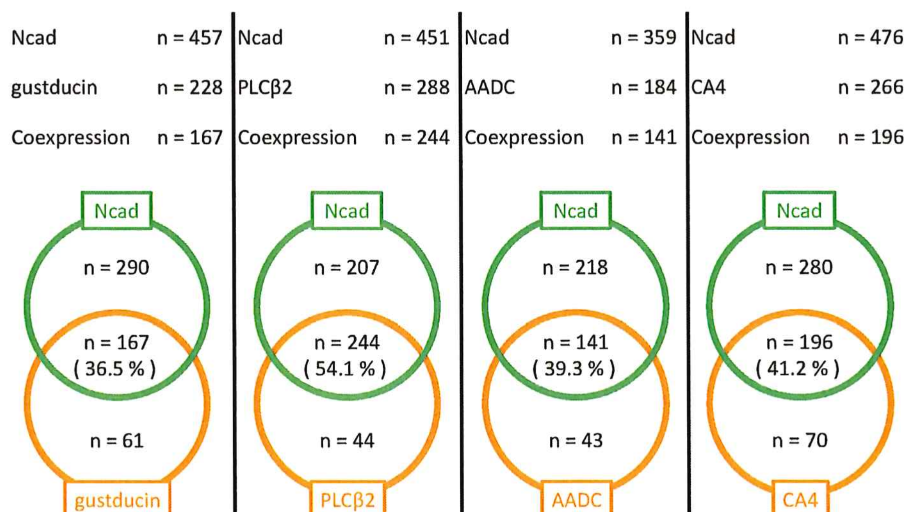

Fig. 5. Venn diagram of N-cadherin and type II or III cell marker expression. The number of taste bud cells expressing N-cadherin and type II or III cell markers is displayed. Cells positive (number and percentage) for both proteins are shown in the overlapped region of the Venn diagram.

speculated that type II and III cells may express different sets of cell surface molecules, and specific combination of surface molecules may allow establishment of proper connections between nerve terminals and taste receptor cells. Our study results indicated that N-cadherin-expressing taste cells are type II and type III cells, which are considered to be taste receptor cells. We found that a large population of N-cadherin positive cells expressed type II cell markers, and approximately 40% of N-cadherin positive cells expressed type III cell markers (Fig. 5). Taste bud cells immuno-reactive for N-cadherin were mostly type II and III cells. A few cells that lacked N-cadherin may be in the process of differentiating from stem cells within the taste buds, or were undergoing apoptosis at the end of their lifespan. Type II cells recognize bitter, sweet and umami receptor cells. Previous studies have shown that type II cells do not make conventional synapses with nerve terminals [21,37]. The fact that type II cells lack conventional synapses with nerve terminals might suggest the existence of alternative mechanisms for transduction of taste information between type II cells and nerve terminals. In combination with results from previous studies, we propose that N-cadherins might play a functional role in correctly establishing nerve terminal connections.

5. Conclusions

In conclusion, the present study clearly demonstrated that subsets of type II and III cells in adult taste buds express N-cadherin. The function of N-cadherin in taste bud cells remains to be revealed. Further studies that target cell surface molecules will provide better understanding of the establishment of functional neural circuits within taste buds.

Ethics approval

Ethics approval was obtained for all experimental procedures.

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Conflict of interest

The authors declare no conflicts of interest.

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