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Sweet Taste Receptors in Normal and Pathological Rat Brain

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1 Introduction

Sugar evokes a distinctive perceptual quality of sweetness and is generally highly preferred. Actually, our sense of taste has the capability of detecting and responding to various tastes, such as sweet, bitter, sour, salty, and umami [1]. Interestingly, the sweet taste receptors, T1Rs, have not only the responsibility to distinguish the sweetness of honey from bitterness of semen armeniacae, but also have been proposed to be associated with the brain glucose sensor recently. Taste-like signaling mechanisms in the brain might be involved in the central regulation of homeostatic processes. Different lines of evidence support the notion that taste signalling molecules might function as chemosensors in different physiological settings.

1.1 Molecular description of sweet taste receptors and their general function

Sweet is one of the main attractive taste modalities in humans. Taste receptor cells (TRCs) in the taste buds receive various substances and transmit gustatory information to the nervous system. Recent advancements in molecular genetics and biochemistry has revealed that each TRC specializes in tuning a single modality of the five basic tastes (umami, sweetness, bitterness, sourness, and saltiness) perceived by humans, and these variations in taste, such as sweetness and bitterness, are caused by activating mutually exclusive subsets of TRCs [1]. Sweet, umami, and bitter TRCs lack conventional synaptic structure [2, 3]. T1Rs are mammalian sweet taste receptors that combine to assemble 2 heteromeric G protein-coupled receptor complexes (GPCRs). The mammalian sweet taste receptors T1r1, T1r2, and T1r3 have been characterized by Nelson and co-workers [4]. They are distantly related to metabotropic glutamate, pheromone, extracellular-calcium sensing

and γ-aminobutyric-acid type B receptors [4–13], which are characterized by the presence of long amino-terminal extracellular domains and considered to mediate ligand recognition and binding [14]. What do these cells do? More than 30 years ago, genetic studies of sweet taste in mice identified a single principal locus that influences and responses to several sweet substances [15, 16]. This locus, known as *Sac*, determines threshold differences in the ability of some strains to distinguish sucrose- and saccharin-containing solutions from water [16]. The *Sac* locus was recently shown by linkage analysis [4, 9–12, 17] and genetic rescue [4] to encode T1R3, thus implicating a member of the *T1r* gene family in sweet taste detection.

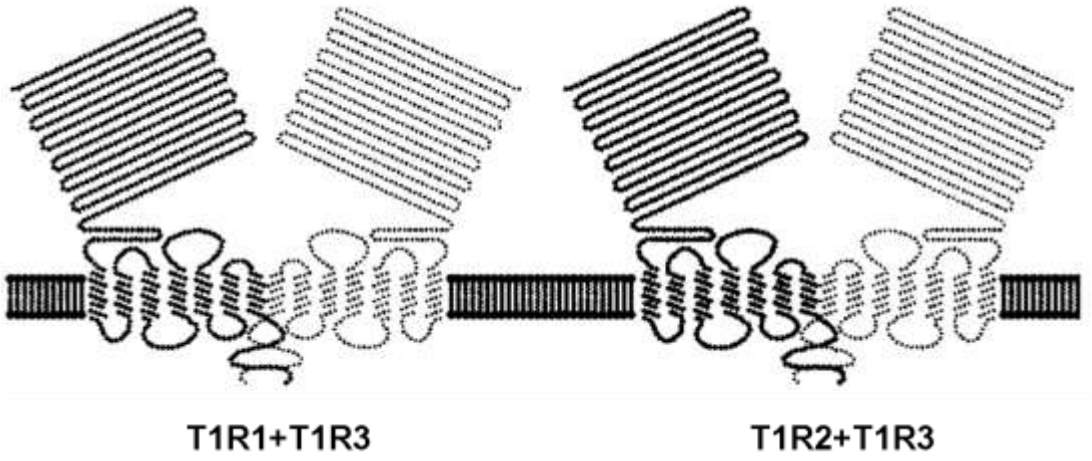

Figure 1 Schematic representation of heteromeric complexes of the sweet taste receptors (T1Rs). (Adapted from Jayaram Chandrashekar et al. [1])

The critical role of T1Rs in sweet taste detection and perception emerged from an ensemble of studies. T1Rs were expressed in subsets of TRCs, and their expression pattern defines three cell types (Figure 1): TRCs co-expressing T1R1 and T1R3 (T1R1+3 cells), TRCs co-expressing T1R2 and T1R3 (T1R2+3 cells) and TRCs containing T1R3 alone [4]. T1R1 is selectively

expressed in subsets of taste receptor cells of the tongue and palate epithelium [5]. It was initially mapped to the distal end of chromosome 4, in the proximity of *Sac* [5]. T1R3 was shown to be expressed in subsets of taste receptor cells in various taste papillae [9-12]. The expression of T1R3 in circumvallate, foliate, fungiform, and palate taste buds showed that T1R3 is expressed in about 30% of cells from all types of taste buds [9, 11]. This topographic pattern of expression closely approximates the aggregate of T1R1 and T1R2 expression [5], and suggests possible coexpression of T1R1 with T1R3 and of T1R2 with T1R3. The coexpression of T1R2 and T1R3 in circumvallate [10, 11] and foliate papillae [11] was recently examined by RT-PCR and in situ hybridization, but a comprehensive study of all three T1Rs in the different classes of taste buds was lacking. Similarly, T1R1 was coexpressed with T1R3 in fungiform and palate taste receptor cells.

1.2 Sweet taste receptors and brain glucosensors

Brain glucosensors are specialized neurons that respond to local fluctuations in extracellular glucose levels, modulating their mean firing rate according to changes in glucose concentration [19, 20]. Early electrophysiological studies described the presence of glucosensing neurons in brain regions known to influence glucose homeostasis, including the hypothalamus and the brainstem [21, 22]. Later studies have identified the presence of glucosensing neurons in other brain regions including the amygdale [23], septum [24] and cortex [25].

Sweet taste signaling is known to be mediated by heterodimeric G-protein coupled receptors and specific downstream signaling elements. More precisely, the transduction of sweet tastants is mediated by the taste genes *Tas1r2* and *Tas1r3* [26], whose T1R2 and T1R3 products assemble to form the heterodimeric sweet receptor T1R2/T1R3 [5, 13, 27]. A similar mechanism mediates the recognition of l-amino acids via the *Tas1r1* and *Tas1r3* genes [26, 7]. T1R1/T1R3 receptor signaling are at least partly supported by the

taste-specific heterotrimeric G-protein gustducin, formed by α-gustducin [28], Gβ3 and Gβ13 [29]. Because the sweet receptor T1R1 or T1R3 is also expressed in the gastrointestinal tract [30], where it plays important physiological roles by mediating hormonal responses to the presence of tastants in the lumen [31], we hypothesized that sweet taste related signaling molecules might also be involved in responses to extracellular levels of brain glucose.

T1R1 and T1R3 proteins are broadly acting sweet taste receptors. They exert their function through two pathways. One is through α-gustducin, a transducin-like G protein a-subunit, to activate adenylyl cyclase, and then leads to an increase in cyclic adenosine 3', 5'-monophosphate (cAMP). The other way is through the α-subunit of gustducin to activate a phospholipase Cβ2-dependent pathway [26, 32, 33]. In addition to the taste receptor cells in the tongue, the sweet taste receptor is expressed in enteroendocrine and pancreatic b cells and has been proposed to be associated with the regulation of glucose absorption and enteroendocrine hormone secretion [31, 34–37]. In an elegant study, Ren and co-workers [38] showed that neurons located in the hypothalamus, hippocampus and cortex express the sweet taste receptors, T1R2 and T1R3, and that food deprivation and hyperglycemia induced significant changes in the expression levels of their sweet-associated genes Tas1r1 and Tas1r2 in the hypothalamus. This suggests that sweet taste receptors are candidates to be associated with the brain glucose receptor.

Glucose is an essential substrate for brain oxidative metabolism and the brain is dependent on a continuous supply of glucose for neuronal function [39]. It also plays an important role in some pathological situations, such as tumor and ischemia. Therefore, we presumed that there might be some correlations between T1Rs and such pathological situations, or T1Rs might play roles in brain tumorigeneses and cerebral ischemia. As we know, C6 rat gliomas proved to be a very reliable model for human glioblastoma growth and invasion [40]. Then we did research on C6 rat glioma and focused on the relationship

between T1Rs and tumor. Ischemic injury results in acute impairment of glucose and energy metabolism. In response to this metabolic stress, there is a need for more glucose because of rapid oxygen depletion and regulation of brain glucose transporters becomes an important factor [41]. Given that sweet taste receptors function as glucose sensors in the brain, as suggested by Ren et al. [39], sweet taste signaling elements might be involved in responses to altered extracellular levels of glucose induced by ischemic injury.

Here we summarized the expression of sweet taste receptor T1R1 and T1R3 in rat normal and pathological brain for the first time.

2 Materials and methods

2.1 Animal experiments

2.1.1 C6 glioma tissue libraries

Rat brains of C6 glioma were obtained from the tissue libraries of department of neuropathology of Tuebingen university [42]. C6 cells were implanted intracranially into rat brains as described [42]. Briefly, cells were harvested and 5 µl of cell suspension (4×10^5 cells/µl) were injected into the basal ganglia region of Sprague Dawley rats. After 14 and 24 days, ten rats were sacrificed, perfused with 50–100 ml of 0.1 M phosphate-buffered saline (PBS) (pH 7.4) containing 4% paraformaldehyde (PFA), and brains were taken for histology. In addition to the rat glioma, three normal rat brains and rat spleens were examined as controls.

2.1.2 MCAO model

2.1.2.1 Establishment of focal cerebral ischemia

Adult male Sprague-Dawley rats weighing 220-280 g were purchased from the Governmental Animal Care Committee at Tongji Medical College and were housed with equal daily periods of light and dark and free access to food and water. All efforts were made to minimize the number of animals and their suffering.

The right middle cerebral artery (MCA) was occluded by the intraluminal suture technique described by Takano [43] with only minor modifications. Using blunt dissection techniques, the right common carotid artery was exposed through a midline cervical incision and carefully dissected free of the vagus nerve under operating microscope. The external and internal carotids were also isolated. The external carotid and the common carotid arteries were

ligated with a 3-0 suture. The pterygopalatine branch of the internal carotid artery was also ligated to prevent incorrect insertion of the embolus. The embolus, silicone-coated 28mm length of a 4-0 monofilament nylon suture (Ethicon), was introduced from the carotid bifurcation into the internal carotid artery until a mild resistance was felt (17 to 19 mm), resulting in occlusion of the origin of the MCA. The embolus was secured in this position using a tight ligature around the CCA.

Post-mortem studies were separately performed 24 h, 3 days, 7 days after MCAO. Rats were deeply anesthetized with ether and perfused intracardially with 4 °C, 4% paraformaldehyde (PFA in PBS). The rat brains were quickly removed and post-fixed in 4% PFA overnight at 4 °C. Brains were cut equally into 3 parts with matrix (as described above). All of these tissue were embedded in paraffin, sectioned serially (3 μm) and mounted on silane-covered slides.

2.1.2.2 Brain sectioning for measurement of T1R1 and T1R3 expression

In each animal, the brain was sectioned into three slices beginning 3 mm from the anterior tip of the frontal lobe (Figure 2). Section B (4 mm thick) was used for measurement of taste receptor expression. Regions from the right and left hemispheres of section B that corresponded to the ischemic core and penumbra were dissected. We initially identified the midline between the two hemispheres and then made a longitudinal cut (from top to bottom) approximately 2 mm from the midline through each hemisphere. This was done to avoid mesial hemispheric structures, which are supplied primarily by the anterior cerebral artery. We then made a transverse diagonal cut at approximately the “2 o’clock” position (as shown in Figure 2) to separate the core (ie, striatum and overlying cortex) from the penumbra (adjacent cortex). Designation of these core and penumbral regions was based on pharmacological and histopathological studies that defined the core to include

subcortical structures, primarily the lateral caudoputamen and overlying cortex, whereas the adjacent ventrolateral cortex was designated as penumbra [44–51]. To compare the T1R1 and T1R3 expression in ischemic region of right hemisphere and non-ischemic region of left hemisphere, we named ischemic core and penumbra region as core^{+}, while the corresponding region as core^{-} and penumbra respectively (Figure 2).

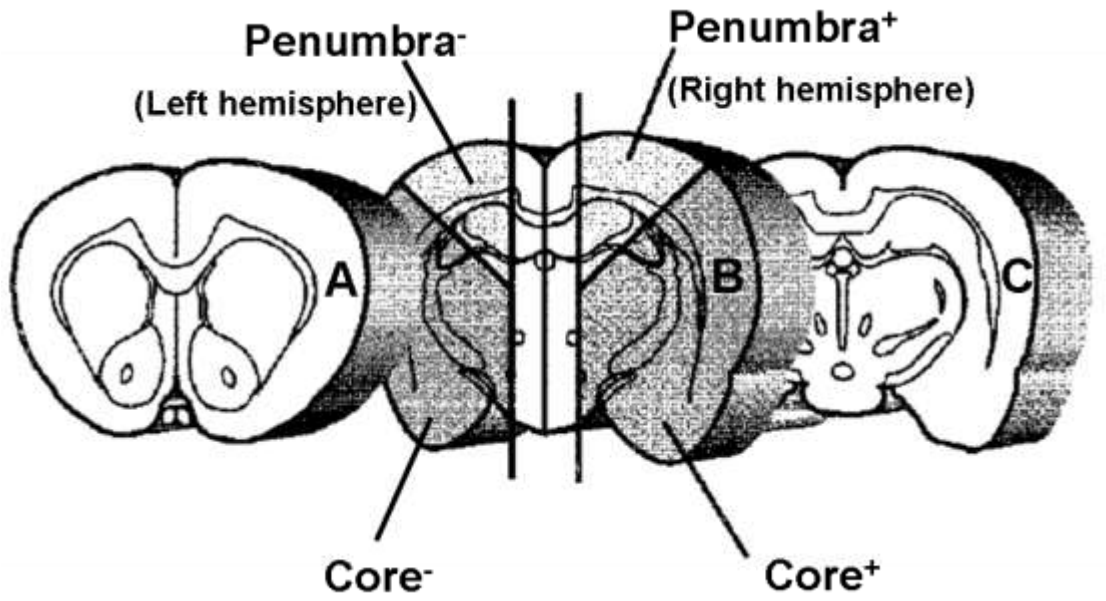

Figure 2 A, C: The anterior and posterior sections (3mm) B: Depiction of the 4 mm coronal section, from which core^{+} and penumbra^{+} in the right hemisphere, core^{-} and penumbra^{-} in the left hemisphere were divided.

2.1.2.3 Measurement of core and penumbral T1R1 and T1R3 expression

After immunostaining, sections from each group were examined by light microscopy and the numbers of TIR1^{+} and TIR3^{+} cells were counted. Positively

stained cell counting based on IHC results has been well developed to semiquantify signal intensity [52]. Positively stained cells were counted by independent observers. To evaluate positive cell numbers in the regions predesignated as “core” and “penumbra” from the right hemispheres. Five microphotos of the “core” and “penumbra” region from the right hemispheres (named $core^+$ and $penumbra^-$ area) were taken under 400× magnification using Nikon Coolscope (Nikon, Düsseldorf, Germany) separately and randomly, and five microphotos of the corresponding region from the left hemispheres ($core^-$ and $penumbra^-$ area). Only positive cells with the nucleus at the focal plane were counted.

2.2 Immunohistochemistry

Immunohistochemistry (IHC) was performed on 3 µm paraffin-embedded sections using antibodies T1R1 (1:200; LifeSpan, Eching, Germany) and TIR3 (1:300; LifeSpan, Eching, Germany) following the steps of diaminobenzidine(DAB) staining. After dewaxing, sections were boiled (in an 850 W microwave oven) for 15 min. in citrate buffer (2.1g citric acid monohydrate/L, pH6) (Carl Roth, Karlsruhe, Germany). Endogenous peroxidase was inhibited by 1% H_2O_2 in pure methanol (Merck, Darmstadt, Germany) for 15 min. Sections were incubated with 10% normal pig serum (Biochrom, Berlin, Germany) to block non-specific binding of immunoglobulins and then with the primary antibodies overnight at 4°C. Antibodies binding to tissue sections were visualized with secondary biotinylated antibodies (goat anti- rabbit or mouse anti- rabbit) (1:400; DAKO, Hamburg, Germany). Subsequently, sections were incubated with a Streptavidin–Avidin–Biotin complex (1:200, DAKO, Hamburg, Germany), followed by development with diaminobenzidine (DAB) substrate (Fluka, Neu-Ulm, Germany). Finally, sections were counterstained with hemalum. As negative controls, the primary antibodies were omitted.

After immunostaining, sections from each group were examined by light microscopy and the numbers of $TIR1^+$ and $TIR3^+$ cells were counted. Positively stained cells were counted by independent observers.

2.3 Statistical analysis

The unpaired t-tests were performed to compare differences between $core^+$ and $core^-$ area, $penumbra^+$ and $penumbra^-$ area of MCAO rats for single time point, and for comparision of each two time points (Graph Pad Prism 4.0 software). Results were calculated as arithmetic means of positive cells per HPF and standard errors of means (SEM). For all statistical analyses, significance levels were set at $p < 0.05$.

3 Results

3.1 Anatomical distribution of immunohistological signal of T1R1 and T1R3 in normal rat nervous system

Immunohistochemistry was used to study the cellular localization and regional distribution of the sweet taste receptors. We have detected the presence of T1R1 and T1R3 in the rat nervous systems. Overall, we found immunohistological staining of T1R1 in rat cortex, hippocampus, hypothalamus and spinal cord obtained by DAB immunostaining (Figure 3). Most intense T1R1 staining was seen in cortex and the signals in cortical were restricted to layer IV. Immunoreactivity for T1R1 could also be seen in neurons in the granule cell and pyramidal cell layers in the hippocampus, and strong signals were observed on the CA fields and dentate gyrus and also the proximity of the hippocampus to the lateral ventricle. In the spinal cord, we found especially high immunohistological signals of T1R1 in the ependymal cells of central canal and in the neurons located in the anterior and posterior horn. Finally, it must be noted that additional areas were found to express taste receptor, especially areas of the periventricular regions around the dorsal aspect of the third ventricle, including the medial habenula and the epithelial cells of the choroid plexus as well as the paraventricular nucleus of the hypothalamus.

The spatiotemporal distribution pattern of T1R3 immunoreactivity closely matched that of T1R1 (Figure 4). Expression of T1R3 was observed in rat cortex, hippocampus, ependyma, choroid plexus and hypothalamus. These results were in agreement with previous findings [38].

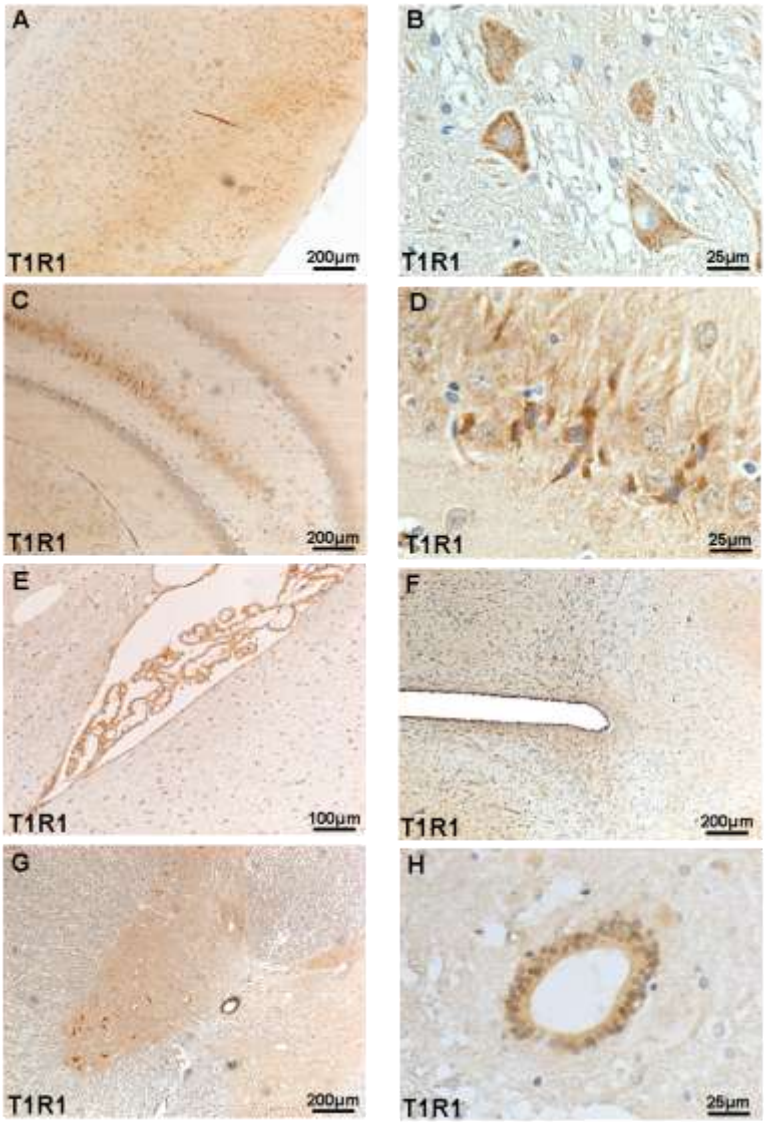

Figure 3 Anatomical distribution of T1R1 in normal rat nervous system

Images of A-H including rat cortex, hippocampus, hypothalamus and spinal cord obtained by DAB immunostaining. A-B, high levels of immunohistological signal in cortex, and the signal seemed to be restricted to layer IV; C-D, the strong signal of T1R1 in hippocampus, the preferential expression of taste-related elements in neurons; E, expression of T1R1 in the paraventricular nucleus the hypothalamus and generally stronger signal of T1R1 in proximity to the third ventricle; F, generally stronger signal in the epithelial cells of the choroid plexus; G-H, strong immunohistological signal of T1R1 in the ependymal cells of central canal and in the neurons of the anterior and posterior horn.

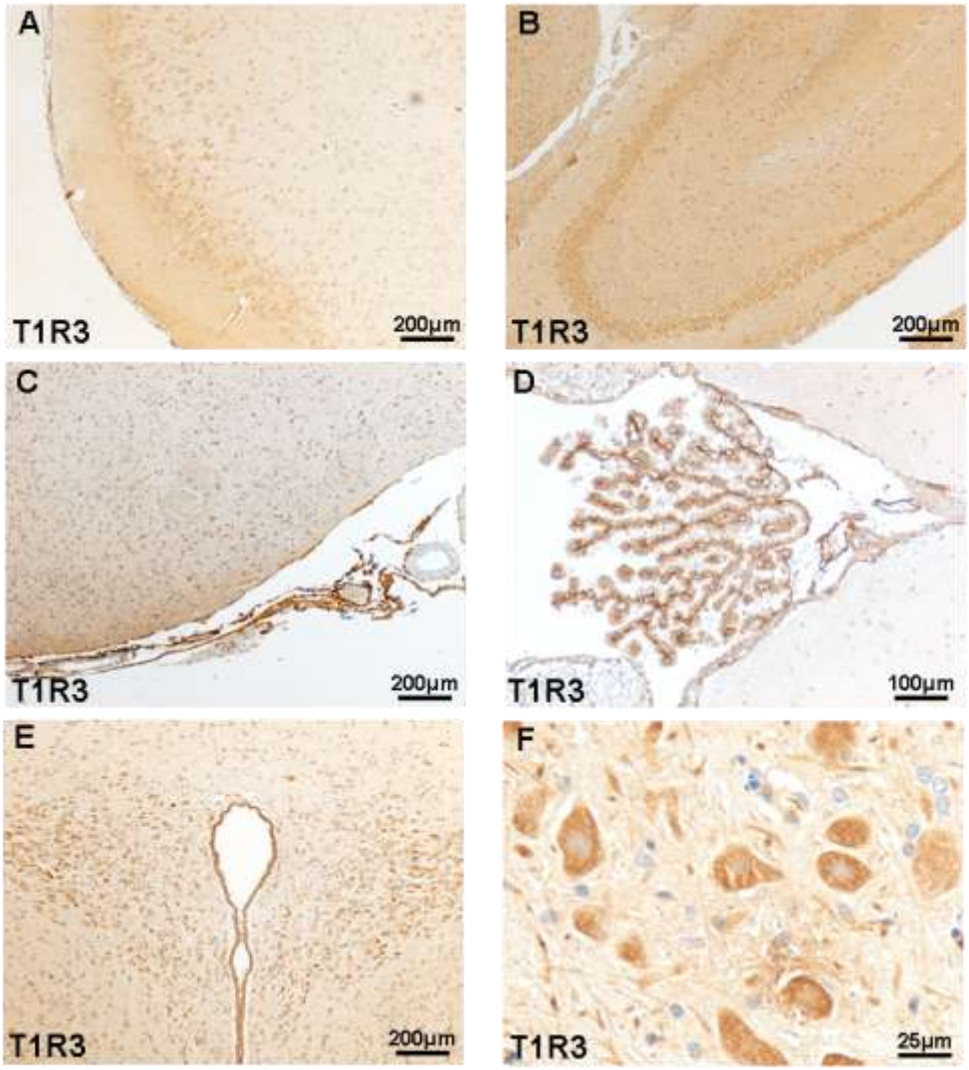

Figure 4 Anatomical distribution of T1R3 in normal rat brain

Images of A-F including rat cortex, hippocampus, ependyma, choroid plexus and hypothalamus obtained from DAB immunostaining methods. A, high levels of expression in cortex, cortical expression was restricted to layer IV; B, high levels of taste receptor T1R3 expression in the hippocampus, the preferential expression of taste-related elements in neurons; C, strong signal of T1R3 in the ependyma; D, generally higher expression levels in the epithelial cells of the choroid plexus; E-F, T1R3 expression in the paraventricular nucleus of the hypothalamus. Note the generally higher intensity of immunostaining of T1R3 in proximity to the third ventricle.

3.2 Immunohistochenistry analyses of T1R1 and T1R3 in rat brains of C6 glioma

To further explore the immunoreactivity of sweet taste receptors in some pathological situation such as tumors, immunohistochemical analyses of T1R1 and T1R3 were respectively performed in rat bains of C6 glioma, especially in the tumor tissue.

The images from rat brain tissue of C6 glioma were obtained by DAB immunostaining methods. Immunohistological signal of T1R1 was observed evidently in the C6 glioma tissue (Figure 5A, 5B, 5C). Strong signal in C6 glioma cells was detected. Meanwhile, signal of T1R1 could also be seen especially clearly in the epithelial cells of ependyma and choroid plexus (Figure 5D, 5E, 5F). The immunoreactivity for T1R3 in the C6 rat glioma closely matched that of T1R1 (Figure 6).

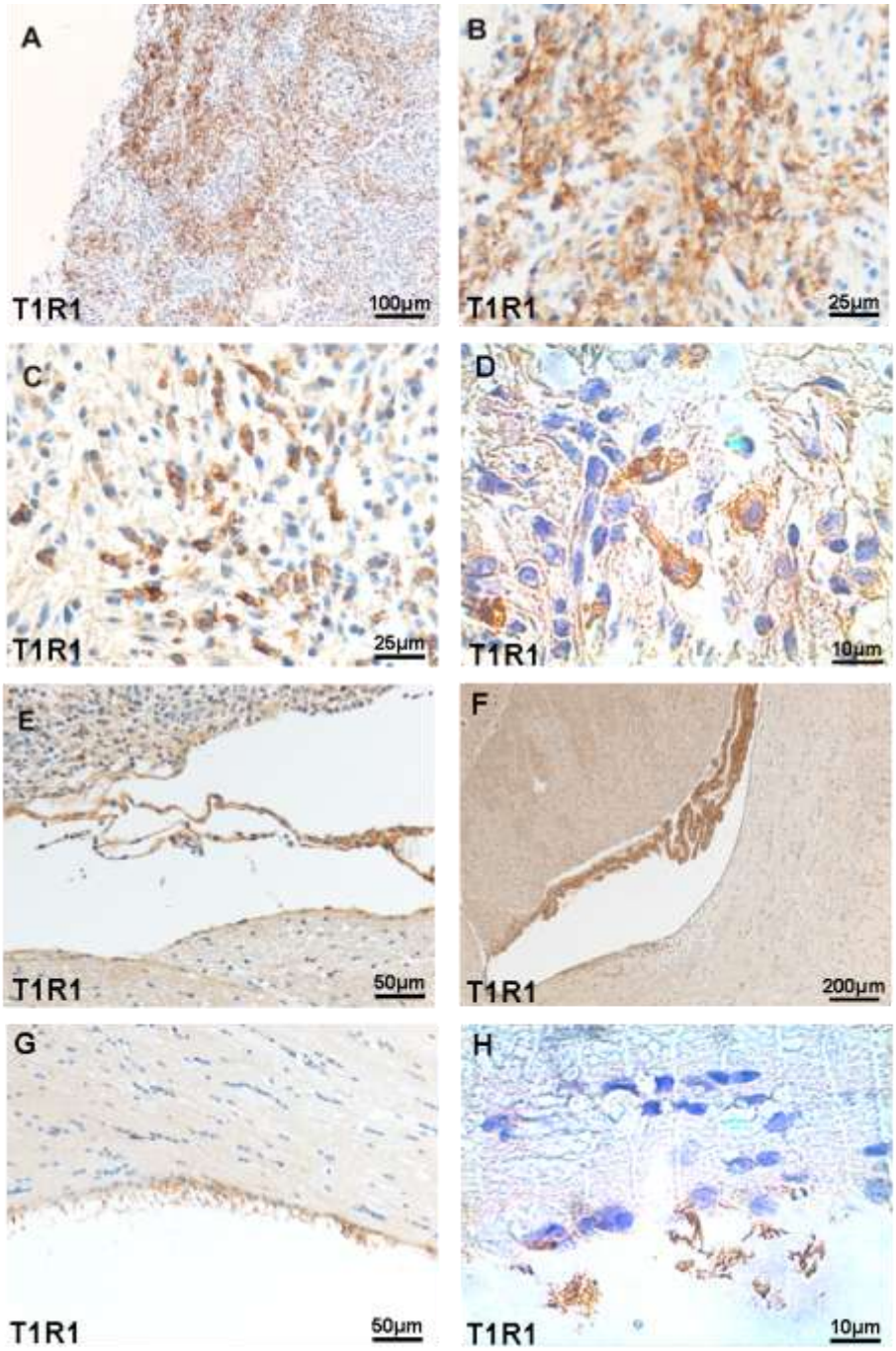

Figure 5 Expression of T1R1 in rat brains of C6 glioma

Images from taste receptor T1R1 immunohistological signal in rat brain tissue of C6 glioma by DAB immunostaining methods. A-D, evident immuohistological signal of T1R1 in the C6 tumor tissue and clear expression in tumor cells. E, T1R1 expression in the epithelial cells of ependyma. F-H, high immunohistological signal in choroid plexus.

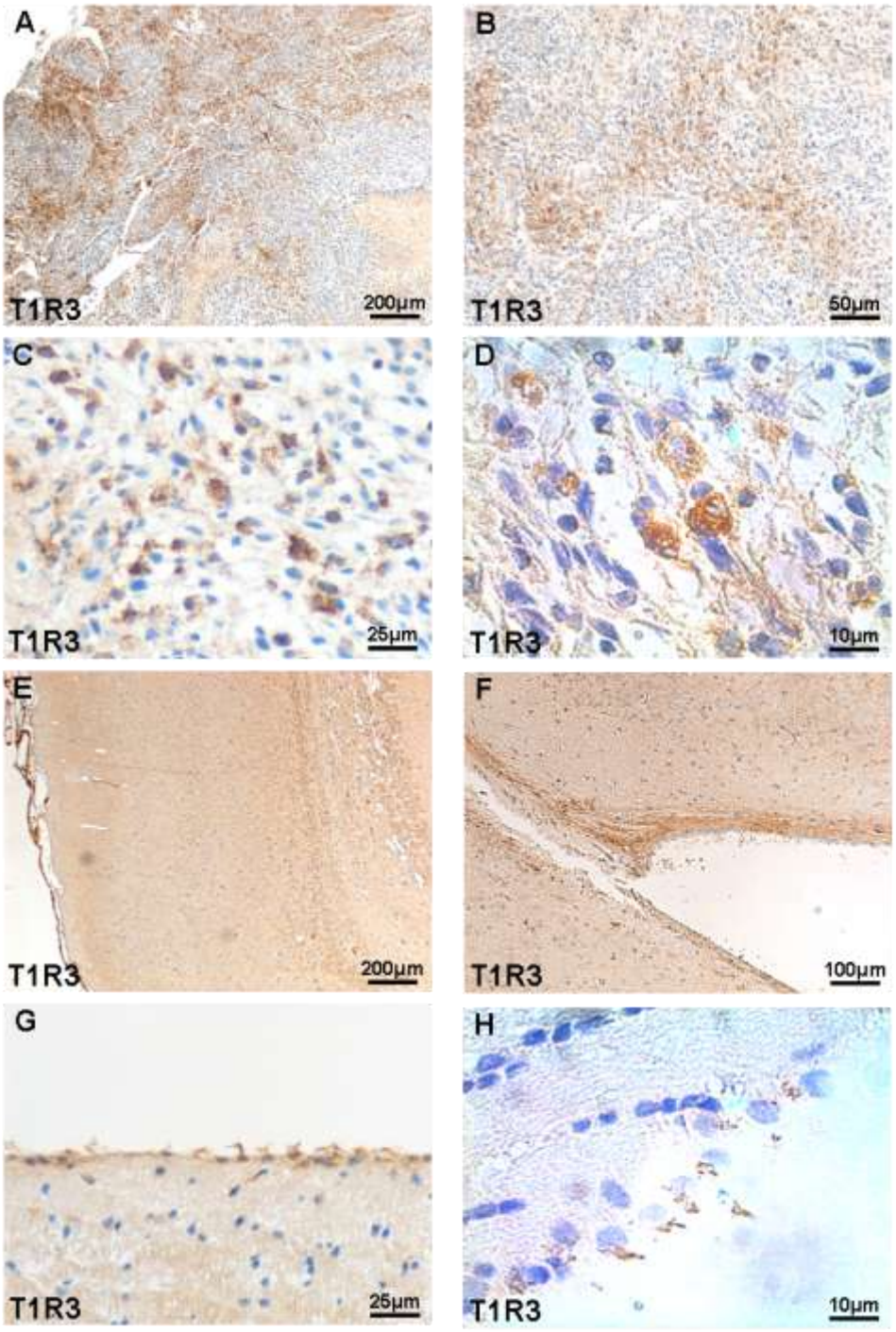

Figure 6 Expression of T1R3 in rat brains of C6 glioma

Images from rat brain tissue of C6 glioma by DAB immunostaining methods. A-D, strong immunohistological signal of T1R3 in the C6 glioma tissue. Note the clear signal in C6 glioma cells; E, strong immunohistological signal of T1R3 in the epithelial cells of ependyma; F-H, high signal in choroid plexus.

3.3 Immunohistochemical analysis of sweet taste receptors T1R1 and T1R3 in ischemic rat brains

Immunohistochemistry was used to study the cellular localization and regional distribution of the sweet taste receptors, T1R1 and T1R3, in the postischemic brain tissue. The semi-quantitive methods were used to analyze the expression of T1R1 and T1R3 in ischemic area （core$^+$ and penumbra$^+$） and control areas (core$^-$ and penumbra$^-$).

3.3.1 Immunohistochemistry analysis of T1R1 in ischemic rat brain

In MCAO rats, immunoreactivity for T1R1 could be seen in neurons in the granule cell and pyramidal cell layers in the hippocampus, and was evident in cerebral cortical neurons, in hypothalamic neurons and also epithelial cells of the choroid plexus as well as the ependyma (data not shown). By day1, day 3 and 7 after MCAO, T1R1 immunoreactivity was increased significantly in the core$^+$ regions compared with the core$^-$ regions, separately (Figure 7). Furthermore, expression of T1R1 was evidently increased at day 7 (28,20 ± 3,40/HPF) after MCAO of the core$^+$ region compared with day 1 (9,07 ± 0,96/HPF) and day 3 (18,33 ± 2,01/HPF) (Figure 8), which indicated the expression of T1R1 increased significantly from day 1 to day 7 after reperfusion in the core$^+$ area.（$p<0,05$）.

The expression of T1R1 in the penumbra area was similar to that in the core area. At day 1, day 3 and 7 after MCAO, T1R1 expression was increased significantly in the penumbra$^+$ regions compared with the penumbra$^-$ regions separately （$p<0,05$）(Figure 9). On day 3 after reperfusion, T1R1 expression was evidently increased in the penumbra$^+$ areas compared with day 1 after reperfusion, while T1R1 expression was evidently increased in the penumbra$^+$

areas at day 7 (19,53 ± 3,46/HPF) compared with day 1 (7,13 ± 0,79/HPF) and day 3 (10,10 ± 1,04/HPF) after reperfusion（p<0,05）(Figure 10).

3.3.2 Immunohistochemistry analysis of T1R3 in ischemic rat brains

In MCAO rat brains, immunohistological signal of T1R3 could also be seen in neurons in the granule cell and pyramidal cell layers in the hippocampus, and was evident in cerebral cortical neurons and in hypothalamic neurons (data not shown), in agreement with previous findings [38]. The immunohistological signal intensity of T1R3 was increased significantly in the $core^+$ regions compared with the $core^-$ regions separately at day1, day 3 and day 7 after MCAO (Figure 11). In addition, the signal intensity of T1R3 was significantly increased on day 7 (30,70 ± 2,74/HPF) after MCAO of the $core^+$ region compared with day 1 (15,87 ± 1,28/HPF) and day 3 (11,80 ± 2,00/HPF)（p<0,05）(Figure 12).

At each time point, the expression of T1R3 was increased significantly in the $penumbra^+$ regions compared with the $penumbra^-$ regions separately (Figure 13, 14) (p<0,05). However, the expression of T1R3 in $penumbra^+$ region was not increased from day 1 to day 7 after reperfusion.

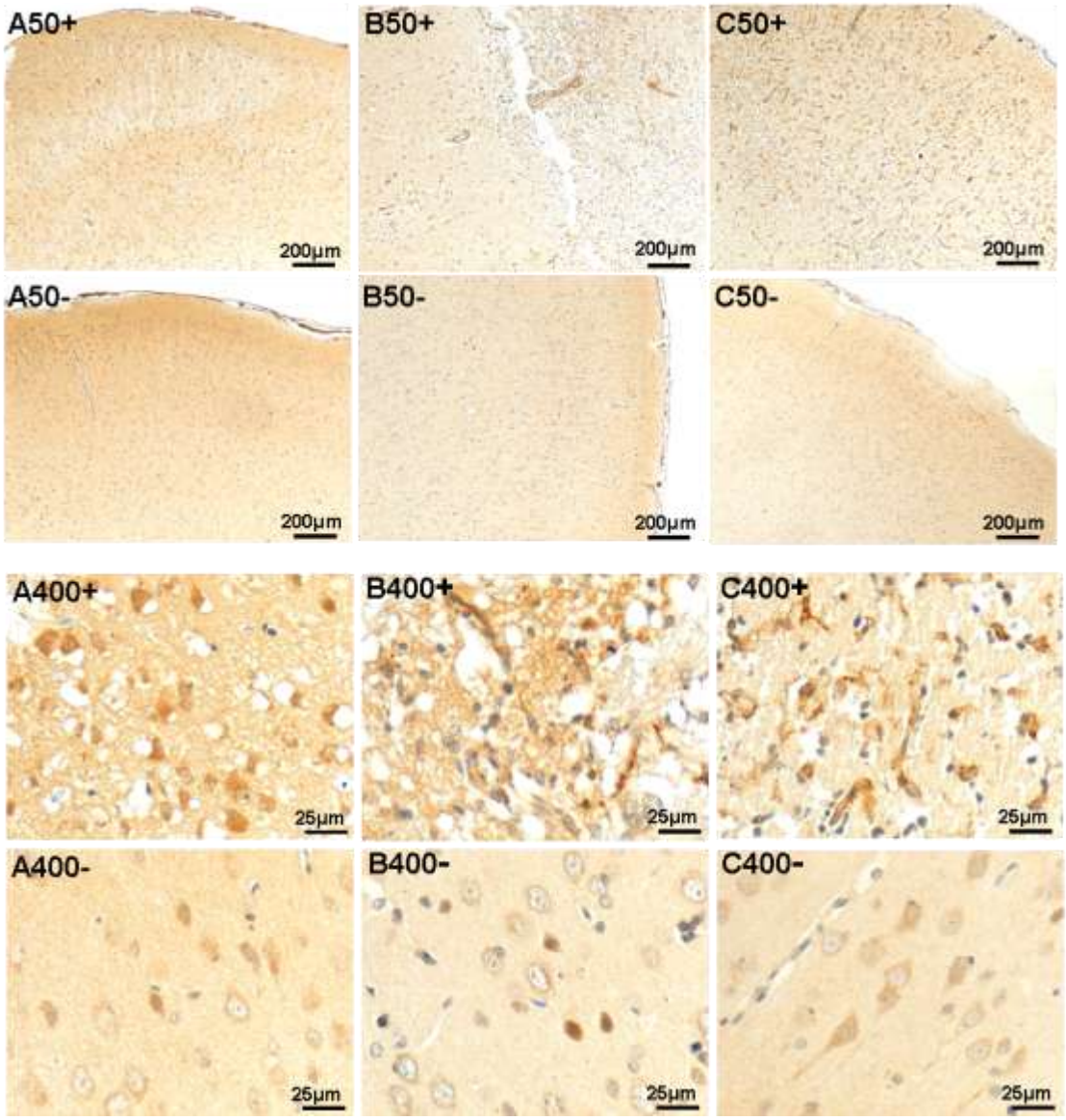

Figure 7 Immunohistochemical analysis of T1R1 in core area at 1 day, 3 days and 7 days after MCAO.

A50+, B50+ and C50+, the location of T1R1 in the ischemic area (named core^{+} area) at 1 day, 3 days and 7 days after MCAO, respectively; A50-, B50- and C50-, the immunohistological signal of T1R1 in the core^{-} area at 1 day, 3 days and 7 days after MCAO, respectively; A400+, B400+ and C400+, the immunohistological signal of T1R1 in the core^{+} area at 1 day, 3 days and 7 days after MCAO, respectively; A400-, B400- and C400-, the expression of T1R1 in the core^{-} area at 1 day, 3 days and 7 days after MCAO, respectively.

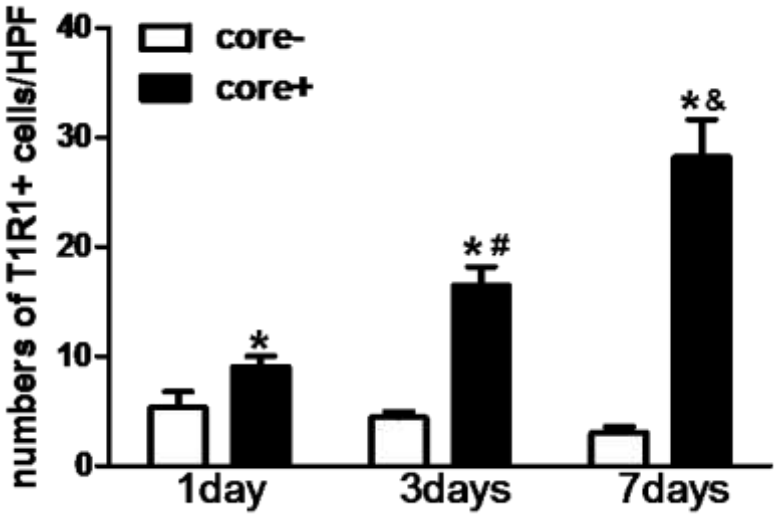

Figure 8 The statistics of T1R1 cells number in core$^+$ and core$^-$ areas.

The unpaired t-test was performed to compare the differences between core$^+$ and core$^-$ for single time point, between two time points in core$^+$ area (Graph Pad Prism 4.0 for Windows). * $p < 0.05$, compared to their respective control core$^-$ area; #, $p < 0.05$, compared to core$^+$ area at day 1 and 7 separately; &, $p < 0.05$, compared to core$^+$ area at day 1 and 3 separately.

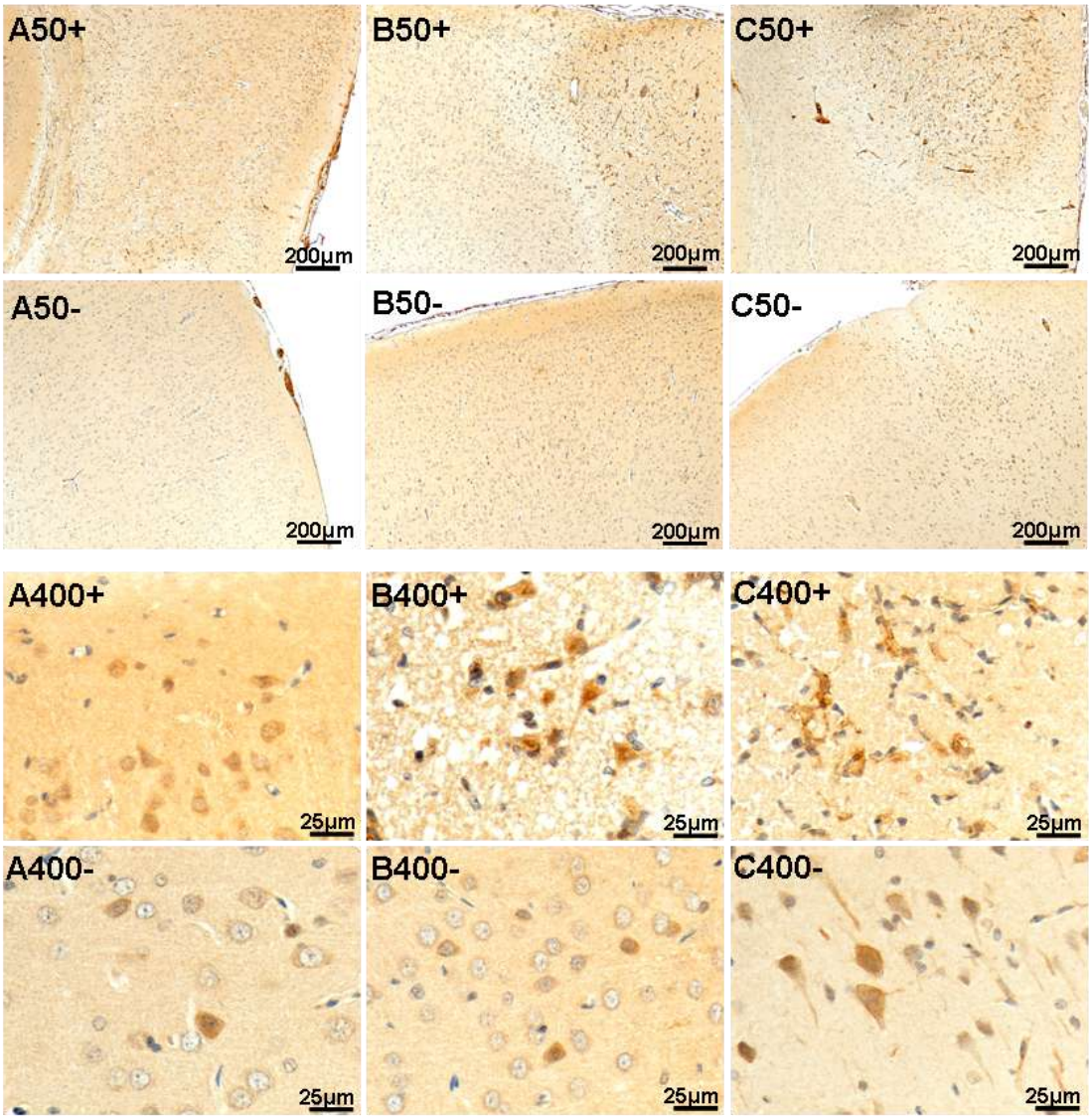

Figure 9 Immunohistochemical analysis of T1R1 in penumbra area at 1 day, 3 days and 7 days after MCAO.

A50+, B50+ and C50+, the immunohistological signal of T1R1 in the penumbra^{+} area at 1 day, 3 days and 7 days after MCAO, respectively; A50-, B50- and C50-, the immunohistological signal of T1R1 in the penumbra^{-} area at 1 day, 3 days and 7 days after MCAO, respectively; A400+, B400+ and C400+, the immunohistological signal of T1R1 in the penumbra^{+} area at 1 day, 3 days and 7 days after MCAO, respectively; A400-, B400- and C400-, the immunohistological signal of T1R1 in the penumbra^{-} area at 1 day, 3 days and 7 days after MCAO, respectively.

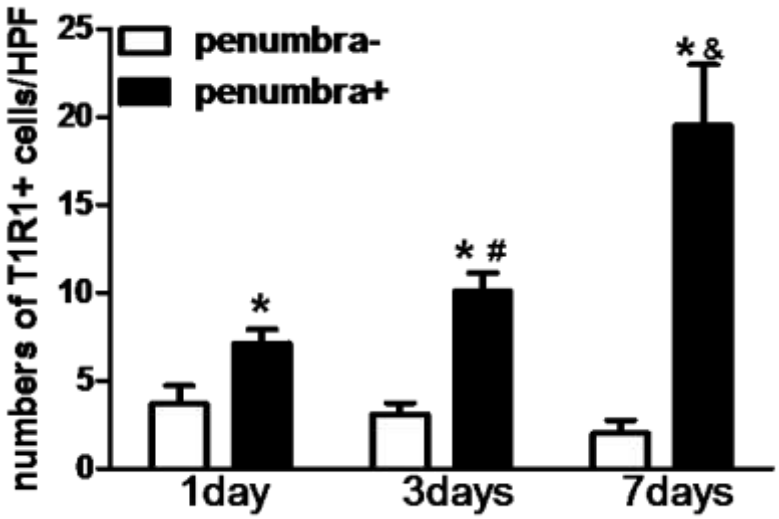

Figure 10 The statistics of T1R1 cells number in penumbra^{+} and penumbra^{-} areas. The unpaired t-test was performed to compare the differences between penumbra^{+} and penumbra^{-} for single time point, between two time points in penumbra^{+} area (Graph Pad Prism 4.0 for Windows). * p < 0.05, compared to their respective control penumbra^{-} area; #, p < 0.05, compared to penumbra^{+} area at day 1 and 7 separately; &, p < 0.05, compared to penumbra^{+} area at day 1 and day 3, separately.

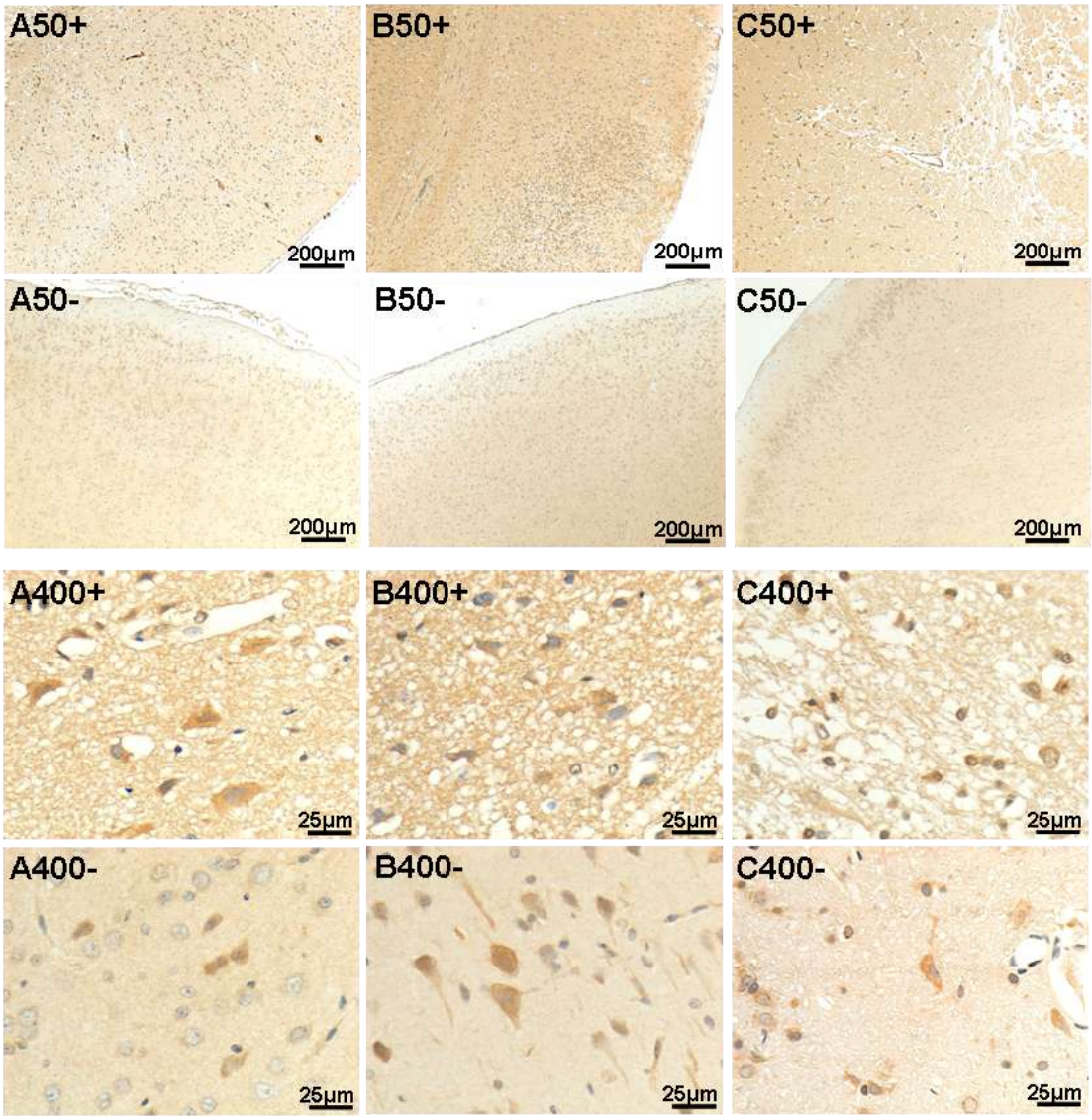

Figure 11 Immunohistochemical analysis of T1R3 in core area at 1 day, 3 days and 7 days after MCAO.

A50+, B50+ and C50+, the immunohistological signal of T1R3 in the ischemic area (named core^{+} area) at day 1 day, 3 days and 7 days after MCAO , respectively; A50-, B50- and C50-, the immunohistological signal of T1R3 in the core^{-} area at 1 day, 3 days and 7 days after MCAO, respectively; A400+, B400+ and C400+, the immunohistological signal of T1R3 in the core^{+} area at 1 day, 3 days and 7 days after MCAO, respectively; A400-, B400- and C400-, the immunohistological signal of T1R3 in the core^{-} area at 1 day, 3 days and 7 days after MCAO.

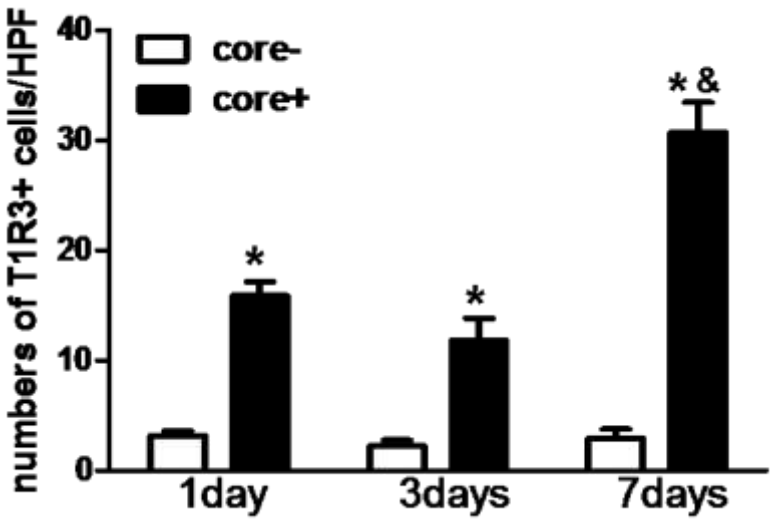

Figure 12 The statistics of T1R3^{+} cells number in core^{+} and core^{-} areas. The unpaired t-test was performed to compare the differences between core^{+} and core^{-} for single time point, between two time points in core^{+} area (Graph Pad Prism 4.0 for Windows). * $p < 0.05$, compared to their respective control core^{-} area; &, $p < 0.05$, compared to core^{+} area at day 1 and 3 separately.

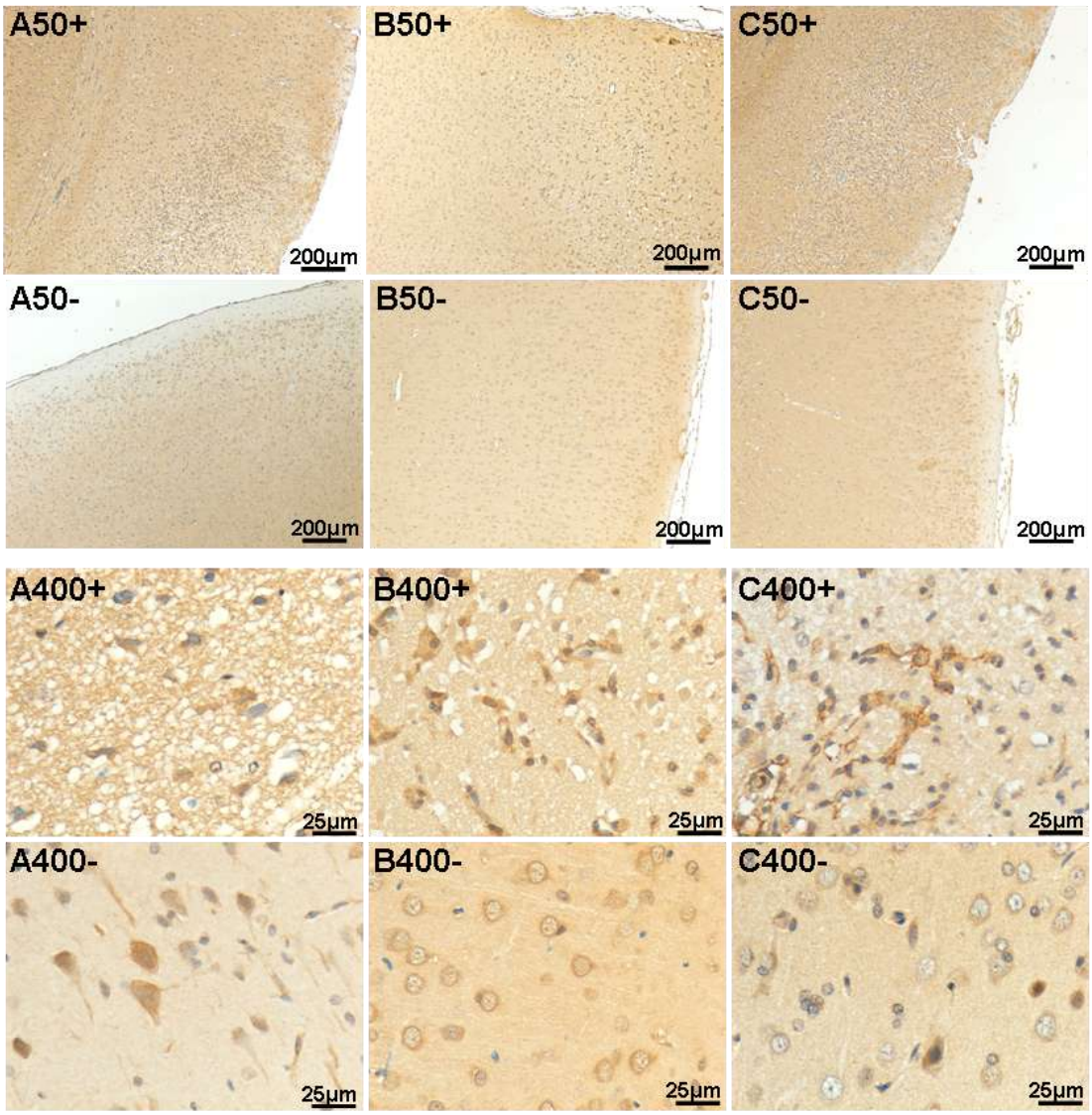

Figure 13 Immunohistochemical analysis of T1R3 in penumbra area at 1 day, 3 days and 7 days after MCAO.

A50+, B50+ and C50+, the immunohistological signal of T1R3 in the penumbra area at 1 day, 3 days and 7 days after MCAO, respectively; A50-, B50- and C50-, the signal of T1R3 in the penumbra$^-$ area at 1 day, 3 days and 7 days after MCAO, respectively; A400+, B400+ and C400+, the immunohistological signal of T1R3 in the penumbra$^+$ area at 1 day, 3 days and 7 days after MCAO, respectively; A400-, B400- and C400-, the signal of T1R3 in the penumbra$^-$ area at 1 day, 3 days and 7 days after MCAO, respectively.

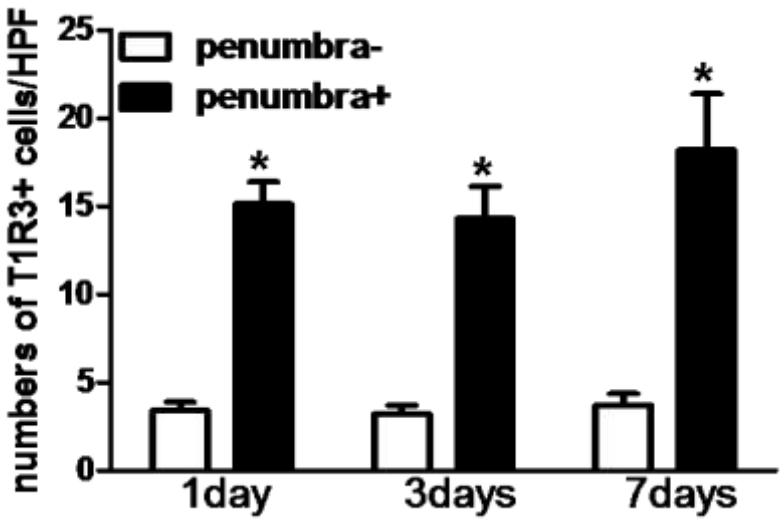

Figure 14 The statistics of T1R3$^+$ cells number in penumbra$^+$ and penumbra$^-$ areas.

The unpaired t-test was performed to compare the differences between penumbra$^+$ and penumbra$^-$ for single time point, between two time points in penumbra$^+$ area (Graph Pad Prism 4.0 for Windows). * $p < 0.05$, compared to their respective control penumbra$^-$ area.

4 Discussion

Here we demonstrated that the expression of sweet taste receptors, T1R1 and T1R3 located in different brain regions, including the cortex, hippocampus, hypothalamus and the habenula. Immuohistological signal of T1R1 was observed in ependymal cells of central canal and in neurons of the anterior and posterior horn in spinal cord as well. We also found the evident signal of T1R1 and T1R3 in the intra-ventricular epithelial cells of the choroid plexus and ependyma. Furthermore, we examined the sweet taste receptors in the rat brain tumor, C6 rat glioma. The immuohistological signal of T1R1 and T1R3 was evidently observed in C6 tumor cells, ependyma and choroid plexus, respectively. Finally, we studied T1R1 and T1R3 in the MCAO rat brain. The immunohistological signal intensity of T1R1 and T1R3 was significantly increased in the ischemic core^{+} and penumbra^{+} areas on day 1, 3 and 7 after MCAO compared with their controls respectively. This is the first demonstration of a change in the expression of the sweet taste receptors, T1R1 and T1R3 in the rat MCAO model. Based on the present results, the immuohistological profiles of T1R1 and T1R3 shared overlapping patterns in normal rat brain, C6 rat glioma and rat brain of MCAO model.

The sweet taste receptors T1Rs have recently been proposed to be associated with the brain glucose sensor [38]. Therefore, taste-like signaling mechanisms in the brain might be involved in the central regulation of homeostatic processes. In fact, different evidences support the concept that taste signalling molecules might function as chemosensors in different physiological settings. The seminal work by Drenckhahn's group provided the first line of evidence that taste-like signalling mechanisms might be used by the gastrointestinal tract to sense the presence of nutrients in the lumen [53]. Their further findings showed that taste mechanisms might also be involved in more general endocrine functions through their expression in the pancreas

[54]. The early indication that such taste mechanisms play important physiological functions agreed with the discovery by Rozengurt and colleagues that the taste G-protein α-gustducin is expressed in PYY- and GLP-1-producing L cells of the intestine [55, 56]. Recent research schows that taste receptor molecules are richly expressed in the intestinal mucosa, including the sweet receptor T1R2/T1R3 [30], where it plays important physiological roles by mediating hormonal responses to the presence of tastants in the lumen [31]. Interestingly, transcript levels of *Tas1r2* are strongly reduced following jejunal glucose perfusion in mice [57], which closely reflects the findings in hypothalamus. Therefore, we propose that an analogous phenomenon takes place with respect to neuronal glucosensing, with similar chemosensory mechanisms acting as glucose receptors in disparate physiological settings. The principle that taste-like signaling mechanisms might play functional roles in the central nervous system also has its precedents. For instance, it has been shown that the tongue-expressed PKD2L1, a polycystic-kidney-disease-like ion channel and candidate mammalian sour taste sensor, is also expressed in specific neurons surrounding the central canal of the spinal cord [58]. More specifically, it has been demonstrated that the PKD2L1-expressing neurons send terminals to the central canal and trigger action potentials in response to decreases in extracellular pH [58]. Among all brain regions, one would expect that nutrient-sensing taste receptors must be found primarily in the hypothalamus, the master brain circuit controlling body homeostasis [59]. Of particular interest was the taste receptors expressed in hypothalamic regions surrounding the ventral aspect of the third ventricle. This privileged anatomical location allows neurons to have proximal access to the contents of the cerebrospinal fluid and to modulate their responses accordingly. Therefore, it is of interest that T1R1 and T1R3 were strongly expressed in the paraventricular nucleus of the hypothalamus, a brain region fundamentally implicated in counter-regulatory responses to hypoglycemia.

The expression of taste sensors in the hippocampus is less likely to have been anticipated. However, several molecular studies demonstrated that the hippocampus is a primary target for ligands that reflect body physiology, including ion balance (calcium, magnesium), insulin, immunity response elements, as well as reproductive, satiety and stress hormones [60]. This argues that a primary, more fundamental role for the hippocampus refers to sensing soluble molecules present in blood and cerebrospinal fluid [60]. We propose that the currently known list of chemosensory functions of the hippocampus must be expanded to include nutrient sensing. The idea that hippocampal neurons have the ability to sense extracellular levels of nutrients is further corroborated by the fact that, in rats, moderate recurrent hypoglycaemia prevented age-related decline in hippocampal-related cognitive functions [61], indicating that hippocampal neurons might functionally detect extracellular levels of glucose in this region. Among these hypothalamic regions, the paraventricular and arcuate nuclei parts have been well investigated. The expressed taste receptor signals in these hypothalamic regions known to be involved in neuroendocrine control, which strongly suggests a role for these molecules as glucose sensors in the brain [62]. A similar observation held for structures such as the habenula and the choroid plexus. Another crucial piece of information deriving from our IHC analyses refered to the neuronal expression of taste receptor proteins. As we know, nutrient sensing in the brain via taste-like signaling pathways might directly effect electrical neuronal activity. This is particularly relevant for the identification of the chemosensors, which have the ability to induce hormone release from neuroendocrine neurons of the paraventricular and arcuate nuclei of the hypothalamus during low glycemic levels. However, it could be argued that such effects depended not on neuronal nutrient sensing, but rather on non-specific systemic effects produced for example by chronic nutrient excess. Further studies could determine the extent to which intracellular glucose metabolism might interact with membrane receptors in regulating neuronal

responses to extracellular levels of glucose. T1R3, unlike T1R1, seems to have the ability to form T1R1/T1R3 homodimers as well [13]. In particular T1R3/T1R3 homodimers display sensitivity to sugars only at relatively high concentrations, e.g., >100 Mm [13], which is certainly outside the limits of physiological glucose concentrations in the brain. Although we can not ascertain, the expression of these dimers was detected on transcript levels in neurons, this is potentially another factor contributing to the insensitivity of *Tas1r3* levels to variations of glucose in the brain at physiological limits. T1R3 has been recently implicated in calcium detection by taste cells [63], because calcium also acts as a nutritional signal in the brain [64].

A similar issue is posed by the lack of significant effects on taste G-protein genes expression levels by changes in nutrient levels. In fact, it appears that all signaling mechanisms downstream to taste G-protein coupled receptors are shared by different classes of chemicals. This holds for the case of both the taste phospholipase PLCβ2 and the non-selective ionic taste channel TRPM5, the deletion of which induces severe impairments in sweet, umami andbitter transduction [27]. Similarly, the G-protein gustducin seems to be important not only for sweet taste signaling, but also for umami and bitter detection [31]. Therefore, and likewise the T1R3 case presented above, the fact that taste G-proteins might be part of the transduction pathways of different taste qualities might have precluded us from detecting changes in these molecules based on analyses of transcript levels. In summary, all these evidence indicated G-protein coupled taste receptors T1R1 and T1R3 might operate as membranebound chemosensors in brain, and in particular that the sweet receptor is a candidate to be associated with the elusive brain glucose receptor.

Several reports have demonstrated that gustducin-coupled sweet taste receptors are not restricted to the gustatory epithelium. They are expressed in enteroendocrine cells and pancreatic β cells, where they sense luminal glucose concentrations and participate in the chemosensory processes

including release of peptides and stimulation of insulin secretion [8, 16, 17]. Ren et al. [38] showed that neurons express sweet taste receptors and a-gustducin and that the hypothalamic expression of taste-related genes is regulated by the nutritional state of the animal. In addition, they showed that expression level of the sweet taste receptor gene was affected by extracellular levels of brain glucose. In a way that is independent from its intracellular metabolism of glucose, which suggests that the sweet taste receptor complex T1R2/T1R3 is a candidate membrane-bound brain glucose sensor. Taken together, these reports strongly suggest that the sweet taste receptor functions as a glucose sensor in tissues other than taste buds in the tongue: i.e., the gut, pancreas and brain. Therefore, we presumed that T1Rs might play important roles in tumorigeneses. As we know, one of the specialities of tumor is growing fast. This speciality is closely associated with glucose metabolism. Expression of T1Rs is also clearly observed in rat C6 glioma tissue，ependyma and intra-ventricular epithelial cells of the choroid plexus. The expression of T1R1 and T1R3 in the tumor cells was evidently increased compared with the normal tissue. These results indicated that sweet taste receptors might play important roles in the tumorigenesis. Further work must be done to solve these questions.

Ischemic injury results in acute impairment of glucose and energy metabolism. In response to this metabolic stress, there is a need for more glucose because of rapid oxygen depletion and regulation of brain glucose transporters becomes an important factor [41]. Given that sweet taste receptors function as glucose sensors in the brain, then we did the research on T1R1 and T1R3 expression in rat brain of MCAO. Constitutive expression of both sweet receptor subunits in neurons of the pyramidal cell and granule cell layers agrees with previously published data [9]. However, we found T1R1 and T1R3 immunoreactivity appeared to be increased significantly in the $core^{+}$ and penumbra regions compared with the $core^{-}$ regions separately by day1, days 3 and 7 after MCAO according to the micrographs obtained from IHC methods.

The upregulation of T1R1 was significantly detected in core and penumbra areas. At 1day, 3 days and 7 days after reperfusion and this reached the maxum at day 7, which coincides with the timing of astrogliosis following ischemic injury [14, 15]. In addition, the expression of T1R1 and T1R3 in increased astrocytes could be observed clearly. All our data showed that the expression of sweet taste receptors was increased in response to ischemic injury.

Several reports indicate that glycerol and lactate could supplement energetic substrates for neurons with the addition of glucose, which is considered the major source of energy [18–21]. In the ‘lactate shuttle’ model, it has been proposed that astrocytes transport and metabolize glucose to lactate that can then be used as a source of energy by surrounding neurons [10, 22, 23]. Cerebral ischemia results in major alterations to oxygen and glucose supplies and cellular metabolism, and a decreased level of glucose in ischemia is believed to play an important role in ischemic injury [24, 25]. In an adaptive response to hypoxia, astrocytes enhance anaerobic glycolysis by an increase in the glycolytic enzyme activity to maintain their cellular energy charge. This plays a critical role in the regulation of brain energy metabolism in both normal and pathologic conditions such as ischemic injury [25–27]. Thus, the induction of sweet taste signalling molecules in the core and penumbra area suggests that their immuohistological signal was up-regulated in response to altered extracellular levels of glucose. It’s caused by ischemic injury in an attempt to maintain the energy supply to the neurons. How the sweet taste receptors function as glucose sensors in the ischemic brain is far from being understood. Several reports have demonstrated an enhancement of glucose transporter expression in response to ischemic injury of the brain [29–32]. Interestingly, the sweet taste receptors and a-gustducin in the intestine function as a luminal sugar sensor to regulate the expression of glucose transporter 2 or Na^+-glucose cotransporter 1 [6, 7]. In addition, the glucose transporter 2 was confirmed as a critical component of the glucose sensor, which needs to be

expressed in astrocytes for the glucose detection system to function [33]. Further, Ve´ga et al. [34] reported astrocytic regulation of glucose transporters to modulate glucose uptake in response to hypoxia. Taken together, the induction of sweet taste signaling molecules in the reactive astrocytes following ischemic injury suggests that they might be involved in the maintenance of glucose homeostasis via the regulation of glucose transporters. However, our study does not provide direct evidence for this or whether these molecules are involved in the mechanisms regulating ischemia-induced altered glucose metabolism. Thus, further studies are needed to investigate the role of sweet taste signaling in brain glucose sensing in astrocytes. Apparently they represent intriguing subjects for evaluating the function of sweet taste signaling in astrocytes at normal brain glucose levels and during high neuronal glucose demand in the future.

In conclusion, we have demonstrated that the expression of sweet taste receptors T1R1 and T1R3 both in normal rat brain and pathological rat brain, C6 rat glioma and rat brain of MCAO model. This study was also the first time to demonstrate of a change in the immuoreactivity of the sweet taste receptors, T1R1 and T1R3 in a rat model of focal cerebral ischemia. The immunoreactivity for T1R1 and T1R3 in the normal rat brain included the neurons in the cortex, hippocampus, hapothalamus and the epithelial cells in the ependyma and chroid plexus. We also found immunohistological signal of T1R1 in the ependymal cells of central canal and in the neurons of the anterior and posterior horn in spinal cord. Strong signal of T1R1 and T1R3 was also observed in the tumor cells of C6 rat glioma. Finally, the immunoreactivity for T1R1 and T1R3 was upregluted in the ischemic area of rat MCAO brain at each time point and increased from day 1 to day 7. These results indicated that ischemic injury might affect the expression of G-protein-coupled sweet taste receptors in rat brain, suggesting that the changed immunoreactivity for sweet taste receptors elicited by cerebral ischemia is involved in glucose homeostasis in the brain. Sweet taste receptors might play important roles in

the mammalian nervous systems.

5 Abstract

The mammalian sweet taste receptors (T1Rs) are G protein-coupled receptor complexes, which have recently been proposed to be associated with the brain glucose sensor. Here, we investigated the expression of sweet taste receptors T1R1 and T1R3 in normal and pathological rat brain, including tissue libraries of C6 rat glioma and rat brain of middle cerebral artery occlusion (MCAO), by immunohistological methods. The results demonstrated that neurons located in different brain regions, including the cortex, hippocampus, hypothalamus and habenula, showed immunohistological signals of T1R1 and T1R3. Additionally, significant T1R1 and T1R3 immunoreactivities were also observed in the intra-ventricular epithelial cells of the choroid plexus and ependyma. Furthermore, immunohistological signals of T1R1 and T1R3 were evidently observed in C6 tumor cells. In addition, we have compared the expression levels of T1R1 and T1R3 in the ischemic core and penumbra areas with non-ischemic areas of the MCAO model. The data showed that the signal intensity of T1R1 and T1R3 was significantly increased in the ischemic areas at day 1, 3 and 7 after MCAO compared with their controls, respectively. Particularly, T1R1 reached the peak level at day 7 (all $p < 0.05$).

To our knowledge, this is the first demonstration of the expression of the sweet taste receptors, T1R1 and T1R3, in C6 rat glioma and rat MCAO ischemia. The present results indicated that ischemic injury might affect the expression of G-protein-coupled sweet taste receptors in rat brain, suggesting sweet taste receptors might play important roles in the physiological and pathological processes in the mammalian nervous system.

6 Zusammenfassung

Die T1R-Proteine sind G-Protein-gekoppelte Rezeptoren, die die Geschmacksqualität „süß“, vermitteln. Vor kurzem wurden die T1Rs in Verbindung mit dem Glukose-Sensor des Säugergehirns gebracht. Wir untersuchten die Expression von T1R1 und T1R3 in normalen und pathologischen Rattengehirnen mittels immunohistologischer Methoden. In unseren Experimenten wurde sowohl gesundes, als auch Gewebe von Hirntumor (Gliom, C6) und MCAO-Läsionen verwendet. Unsere Ergebnisse deuten auf ein unterschiedliches Expressionsnievau von T1R1 und T1R3 in verschiedenen Gehirnregionen hin, insbesondere in der Hirnrinde, im Hippocampus, Hypothalamus und der Habenula. Außerdem konnte die erhöhte Expression von T1R1 und T1R3 auch in den intraventrikulären Epithelzellen des Plexus choroideus und im Ependym nachgewiesen werden. Darüber hinaus haben wir auch die erhöhte Expression von T1R1 und T1R3 in C6 Tumorzellen beobachtet. Im MCAO-Modell ließ sich nachweisen, daß die Expression von T1R1 und T1R3 deutlich in den ischämischen Bereichen an den Tagen 1, 3 und 7 nach Reperfusion erhöht war, wobei die höchste Expression von T1R1 am 7 Tag nach der Reperfusion festgestellt werden konnte ($p < 0,05$).

Unsere Experimente liefern den ersten Nachweis einer Veränderung in der Expression von T1R1 und T1R3 in C6 Ratten Gliomen und im MCAO Modell. Die vorliegenden Ergebnisse zeigen, dass in Folge der ischämischen Schädigung die Expression von G-Protein-gekoppelte Rezeptoren verändert werden kannt. Unsere Ergebnisse deuten darauf hin, dass T1R eine Rolle in den physiologischen und pathologischen Prozesse im ZNS der Säugetiere haben.

7 Abbreviations

cAMP	cyclic adenosine 30,50-monophosphate
CCA	Commen carotid artery
CRF	corticotrophin releasing factor
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
GLP1	glucagon-like peptide 1
GPCRs	G protein-coupled receptor complexes
HPF	High power Field
IHC	Immunohistochemistry
MCA	middle cerebral artery
MCAO	middle cerebral artery occlusion
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PFA	paraformaldehyde
pH	potential of hydrogen
PKD2L1	Polycystic kidney disease 2-like 1
PLCβ2	phospholipase C, beta 2
PVH	proximity to the third ventricle
PYY	Peptide YY
RT-PCR	reverse transcription -Polymerase Chain Reaction
SEM	standard error of the mean
TRCs	Taste receptor cells
T1R1	Taste receptor, type 1, member 1
T1R3	Taste receptor, type 1, member 3

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9 Poster

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Sweet Taste Receptors in mammalian brain

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Our sense of taste is capable of detecting and responding to sweet, bitter, sour, salty, and umami stimuli. T1Rs are mammalian sweet taste receptors that combine to assemble 2 heteromeric G protein-coupled receptor complexes. Nelson et al. (2001) characterized the mammalian sweet taste receptors T1r1, T1r2, and T1r3. Here we summarized the sweet taste receptor in mammalian normal and pathological brain for the first time. Neuronal expression of taste genes was detected in different nutrient-sensing forebrain regions, including the paraventricular and arcuate nuclei of the hypothalamus, the CA fields and dentate gyrus of the hippocampus, habenula, and cortex. Expression was also observed in the intra-ventricular epithelial cells of the choroid plexus. Furthermore, we examined the sweet taste receptors in the rat brain tumors, such as C6 glioma. T1Rs expressions were evidently observed in c6 glioma tissue and also in ependyma, choroid plexus. Xueying Ren et al (2009) investigated that sweet receptor is a candidate membrane-bound brain glucosensor and essential for normal body glucose homeostasis and neuronal function. Then, we presume that T1Rs may play important roles in occurrence, development and lapse of tumors.

Mammalian Sweet Taste Receptors

Figure 1 Schematic representation of sweet taste receptors. T1Rs, may function as heteromeric complexes. Jayaram Chandrashekar, Mark A. Hoon et al. The receptors and cells for mammalian taste. NATURE Vol 444,288-294

Taste-receptor cells, buds and papillae

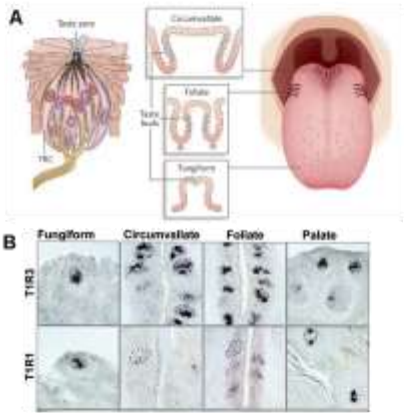

Figure 2 A. Schematic representation of taste buds (left) , circumvallate papillae , foliate papillae and fungiform papillae. TRCs project microvillae to the apical surface of the taste bud, where they form the 'taste pore'; this is the site of interaction with tastants. Jayaram Chandrashekar, Mark A. Hoon, Nicholas J. P. et al., The receptors and cells for mammalian taste, NATURE Vol 444,288-294. B. Expression of T1Rs in Subsets of Taste Receptor Cells In situ hybridizations with digoxigenin-labeled antisense RNA probes demonstrated that T1R3 is expressed in subsets of mouse taste receptor cells (upper panels). Greg Nelson, Mark A. Hoon et al. Mammalian Sweet Taste Receptors. Cell, Vol. 106, 381–390

Co-expression of T1Rs in Vitro cultured embryonic mouse hypothalamic cell line N38

Figure 3 In vitro study by immunofluorescence methods. Confocal images (× 10 magnification) of N38 cells that we determine endogenously express taste-related molecules. A. Expression of the receptor subunit T1R2; B. expression of the receptor subunit T1R3; C. merging, showing that these cells express both subunits of the sweet receptor T1R2/T1R3. Xueying Ren, Ligang Zhou et al. Sweet taste signaling functions as a hypothalamic glucose sensor. Frontiers in Integrative Neuroscience,Vol.3,1-15

Anatomical distribution of the gene Tas1r3

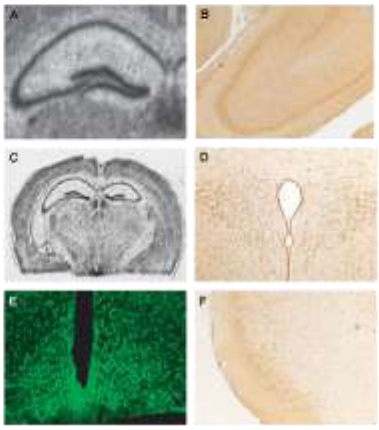

Figure 4 Images of A,C,E (mouse brain) and B,D,E (rat brain) including hippocampus, hypothalamus and cortex obtained from both in situ hybridization and DAB immunolabeling methods were placed alongside. A-B, Note the strong levels of both gene and protein expression on the CA fields and dentate gyrus, the preferential expression of taste-related elements in neurons. The proximity of the hippocampus to the lateral ventricle. C, Overall view of a brain section hybridized against a Tas1r3 probe. Note the high levels of expression in hippocampus, hypothalamus, cortex, habenula and the epithelial cells of the choroid plexus. D, generally higher expression levels in proximity to the third ventricle(PVH). E, T1R3 expression in the arcuate nucleus of the hypothalamus as revealed by immunofluorescence methods (confocal microscope). F, cortical expression seems to be restricted to layer IV. A,C,E from Xueying Ren, Ligang Zhou et al. Sweet taste signaling functions as a hypothalamic glucose sensor, Frontiers in Integrative Neuroscience,Vol.3,1-15

Expression of T1R1 in C6 rat Brain

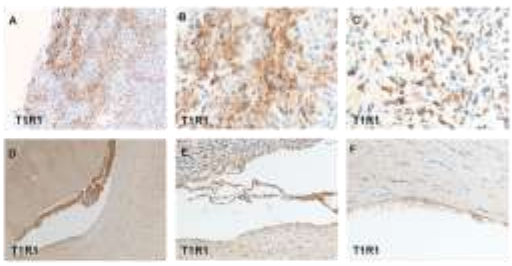

Figure 5 Images from rat brain tissue of C6 glioma with DAB immunolabeling methods. A-C, T1R1 expression evidently in the c6 tumor tissue (A, × 5 magnification; B,C, × 20 magnification). Note the clear expression in C6 glioma cells. D, high level of expression in choroid plexus. E-F, T1R1 expression in the epithelial cells of ependyma.

Conclusion and Outlook

1. Sweet taste receptors express in brain and the anatomical distribution includes forebrain regions: paraventricular and arcuate nuclei of the hypothalamus, the CA fields and dentate gyrus of the hippocampus, the habenula, cortex, intra-ventricular epithelial cells of the choroid plexus.
2. Expression of T1Rs is also clearly observed in rat C6 glioma tissue, ependyma and intra-ventricular epithelial cells of the choroid plexus.
3. Brain glucosensing is essential for normal body glucose homeostasis and neuronal function. However, the exact signaling mechanisms involved in the neuonal sensing of extracellular glucose levels remain poorly understood.Taste receptors might operate as membranebound chemosensors in brain, and in particular that the sweet taste receptor is a candidate to be associated with the elusive brain glucose receptor. Is there correlation between sweet taste receptor and blood glucose in brain?
4. Is there correlation between taste receptor and tumorigenesis, development and lapse?

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11 Curriculum Vitae

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