

# Effects of sucralose on learning and memory in *Caenorhabditis Elegans*

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**Abstract.** Sugar-substituted beverages, including those sweetened with sucralose, are popular as sugar-free alternatives. However, the impact of sucralose on learning and memory abilities remains inconclusive. This study aimed to investigate the effects and mechanisms of sucralose, a commonly used sugar substitute, on the learning and memory abilities of *Caenorhabditis elegans*. Three different concentrations of sucralose were administered to *C. elegans* at the developmental stage to observe the effects on their non-associative and associative learning abilities. Non-associative learning was assessed through tap stimulus and odor chemotaxis assays, while associative learning was measured using a combination of high NaCl concentration and starvation-induced chemotaxis response. RT-qPCR analysis was employed to detect changes in the expression of 11 learning and memory-related genes in *C. elegans* exposed to sucralose, and homology analysis was conducted to compare these genes with their human counterparts. The results showed that *C. elegans* treated with a high concentration of sucralose exhibited significantly prolonged withdrawal reaction times, while those treated with a low concentration displayed reduced odor chemotaxis. Additionally, nematodes treated with different sucralose concentrations demonstrated impaired associative learning ability. RT-qPCR analysis revealed a significant down-regulation in the relative expression of all genes following high sucralose treatment, with the glutamate receptor signaling pathway being the most affected. Homology analysis indicated that 10 out of the 11 genes had homologs in humans. In conclusion, this study suggests that high concentrations of sucralose can diminish the learning and memory abilities of nematodes by extensively modulating learning and memory-related pathways, particularly affecting the glutamate receptor signaling pathway.

**Keywords:** Sugar Substitutes, Sucralose, *C. Elegans*, Learning, Memory.

## 1. Introduction

With the increasing prominence of health problems caused by excessive intake of added sugar, reducing and controlling sugar has become one of the most urgent dietary needs for people [1, 2]. Sugar substitutes are widely favored by consumers due to their high sweetness and low calorie properties [3]. These substitutes can be broadly categorized into three types: sugar alcohols, natural sweeteners, and artificial sweeteners. Erythritol, a representative sugar alcohol, has a sweetness of about 60%-80% of sucrose and a caloric value of only 0.4 kcal/g [4]. Steviol glycoside, another widely used natural sweetener, is approximately 250-350 times sweeter than sucrose and is metabolized into steviol and excreted in the urine [5, 6]. Common artificial sweeteners include aspartame and sucralose. Aspartame is approximately

180-200 times sweeter than sucrose [7], while sucralose is 600 times sweeter than sucrose [8]. Both aspartame and sucralose do not enter the human bloodstream [9]. The demand for sugar-substituted beverages has significantly increased in recent years, as consumers shift towards healthier dietary habits [10]. In China, the market for sugar substitutes has been steadily growing, reaching a size of approximately 22 billion dollars in 2021, with a compound annual growth rate (CAGR) of 35% [11]. However, along with the popularity of sugar substitutes, concerns about the long-term consumption of these products have emerged. Existing studies have shown a positive correlation between erythritol intake and the risk of cardiovascular disease (CVD) [12]. On July 14, 2023, the World Health Organization (WHO) classified aspartame as a Group 2B carcinogen, highlighting potential health risks [13]. Consequently, the safety of sugar substitutes has become an increasingly studied topic, as the existing health concerns are being addressed and further risks associated with these substitutes need clarification.

Learning and memory are crucial survival skills for human beings, with adolescence being a prime time for developing these functions as the nervous system undergoes significant developmental changes [5]. During this stage, continuous learning and exercise lead to the addition of new neurons in the granular cell layer of the dentate gyrus in the hippocampus, contributing to memory formation. When there is nerve damage, it can affect hippocampal function and result in symptoms of memory deficits in adolescents [14]. Several studies have indicated the potential neurotoxicity of sugar substitutes [15-17]. For instance, long-term consumption of sugar substitutes has been found to severely impair passive avoidance learning, affect cognitive and hippocampal integrity, and lead to difficulties in forming long-term memories in rats [15]. Lebda et al. [16] conducted a two-month intervention experiment in which three groups of mice were daily fed water, aspartame, and cola [16]. The results showed significant brain damage in the mice that ingested aspartame and cola, with the mechanism involving circulatory system imbalance and neuronal apoptosis. Using adult zebrafish as a model, Li et al. investigated the behavioral and neurological effects of aspartame intake within the permissible daily intake range, revealing that it altered the behavioral characteristics of zebrafish and disrupted neurotransmitter homeostasis in the brain [17]. Although Finn and Lord's study suggested that sucralose did not induce central nervous system lesions in mice and marmosets, there is still insufficient evidence to draw a definitive conclusion regarding the impact of sucralose on learning and memory functions in organisms [18].

In this study, *Caenorhabditis elegans* was employed as a model organism. *C. elegans* are multicellular invertebrates with transparent bodies, which facilitate experimental observation, making them commonly used for studying learning and memory functions. The nervous system of *C. elegans* is relatively simple, consisting of only 302 neurons, and the structure and connections of these neurons have been extensively described, enabling easier investigation of neural development and circuit formation. Due to their short life cycle and high reproductive capacity, *C. elegans* are highly suitable for large-scale behavioral experiments and genetic studies. Moreover, *C. elegans* share 60-80% genetic homology with humans, making them suitable for targeting conserved genes and molecular pathways [19]. Learning memory behavior in *C. elegans* involves both non-associative and associative learning, allowing them to respond effectively to a single stimulus and establish connections between multiple stimuli [20]. Rankin et al. developed a method to study the non-associative learning ability of nematodes using mechanical stimuli [21]. Additionally, the associative learning of *C. elegans* can be investigated through odor or chemotaxis experiments combined with starvation [22, 23]. Rashmi et al. confirmed the significance of sleep for learning and memory in *C. elegans* through a chemotaxis experiment involving the odor of butanone [24]. Furthermore, Vishnu et al. identified the crucial role of dopamine in the olfactory adaptive learning pathway of *C. elegans* [25]. Therefore, in this study, the tap stimulus assay and odor chemotaxis assay were employed to assess the non-associative learning ability of *C. elegans*, while chemotaxis experiments involving high concentrations of NaCl, combined with starvation, were utilized to examine their associative learning ability.

In this study, it was found that sucrose treatment reduced the non-associative learning ability and associative learning ability of *C. elegans* to a certain extent. The study investigated the impact of sucrose treatment on the learning memory function of *C. elegans*. Different concentrations of sucrose were

examined to evaluate their effects on non-associative and associative learning behaviors of *C. elegans*. Additionally, the study analyzed the expression of 11 well-known genes associated with learning memory using RT-qPCR. The findings revealed that sucrose modulates learning memory pathways by extensively regulating pathways related to learning and memory. Notably, the glutamate signaling pathway was identified as one of the pathways affected by sucrose treatment. This study lays the foundation for further in-depth research on the regulation of learning memory pathways by sugar substitutes in mammals, and suggests the potential harm of sugar substitutes consumption on learning memory ability in developing adolescents.

## 2. Materials and Methods

### 2.1. Experimental Materials

Sucralose (BR, HPLC  $\geq 98\%$ ) was obtained from Shanghai Yuanye Biotechnology Co. Ltd. Benzaldehyde (AR, GC  $> 98.5\%$ ) was purchased from Shanghai Macklin Biochemical Technology Co., Ltd. Peptone, yeast dipping powder, and tryptone were acquired from Beijing AOBOX Biotechnology Co., Ltd. The remaining reagents were purchased from Beijing Solarbio Science & Technology Co., Ltd.

Wild-type *Caenorhabditis elegans* N2 and *Escherichia coli* OP50 strains were obtained from the Chinese Institute of Science and Biophysics.



**Figure 1.** *Caenorhabditis elegans* observed under a stereomicroscope, with different growth stages indicated by arrows. The larval stages 1 to 4 (L1-L4) and the first day of adulthood (D1) are specifically highlighted. Scale bar = 250  $\mu\text{m}$ .

### 2.2. Experimental Methods

**2.2.1. Design of market research forms and implementation.** Through field observation of supermarkets in Beijing, China, we identified the mainstream sugar substitute beverages on the market and recorded data such as manufacturer, beverage name, sugar substitute category, and name of sugar substitute.

**2.2.2. Culture and synchronization of *C. elegans*.** The nematode growth medium (NGM) was prepared by first weighing 0.75 g of NaCl, 0.625 g of tryptone, and 5.0 g of agar powder. The weighed ingredients were then dissolved and the volume was adjusted to 250 mL using deionized water. The medium was sterilized at 115 °C for 30 minutes and subsequently cooled to approximately 65 °C at room temperature. To the cooled medium, 0.25 mL of 1 M  $\text{CaCl}_2$ , 0.25 mL of a 5 mg/mL cholesterol ethanol solution, 0.25 mL of 1 M  $\text{MgSO}_4$ , and 6.25 mL of 1 M  $\text{K}_3\text{PO}_4$  were added, and the mixture was thoroughly mixed. NGM plates were made using the prepared medium, which were then allowed to cool and coated with 100  $\mu\text{L}$  of *E. coli* OP50 as the food source.

*C. elegans* were inoculated on NGM medium and cultured at 20 °C until egg-conceiving adults were obtained. Next, the *C. elegans* were treated with 1% sodium hypochlorite and 0.5 M potassium hydroxide. Embryos were collected during incubation. The embryos were then washed three times using

M9 buffer and transferred onto a restriction medium that was devoid of tryptone and *E. coli* OP50. Following incubation, the *C. elegans* experienced stagnation under conditions of food deprivation. They were subsequently collected and inoculated with NGM medium containing food to initiate normal growth and development, effectively achieving synchronization of the growth cycle of *C. elegans*.

**2.2.3. Effect of sucralose on tap stimulus in the *C. elegans*.** According to the method described by Rankin et al [26], *C. elegans* synchronized to the L1 stage were transferred to different groups of NGM medium. These groups included a control group without any substituted sugar (0 mg/mL), as well as groups with sucralose concentrations of 0.3 mg/mL, 1 mg/mL, and 10 mg/mL. Additionally, there was a non-substituted sugar control with 10 mg/mL of glucos. The *C. elegans* were then incubated for 24 hours.

After incubation, individual *C. elegans* were carefully transferred to new petri plates containing the respective groups of NGM medium (35 mm in diameter). The plates were incubated overnight. On the following day, a needle was used to tap the edge of the petri plate corresponding to the head of the *C. elegans*, and their evasive response to the tap stimulus was observed. The time it took for the *C. elegans* to recover their movement was recorded. This process was repeated several times until the *C. elegans* no longer evasively responded to the tap. If the *C. elegans* still exhibited an evasive response after 10 taps, the time from the start of the evasive response to the recovery of movement on the tenth tap was recorded as the final state.

**2.2.4. Effect of sucralose on odor chemotaxis in the *C. elegans*.** According to the method described by Wang et al [27], *C. elegans* synchronized to the L1 stage were transferred to NGM medium in each group and incubated at 20 °C for 48 hours. Adult *C. elegans* that did not harbor eggs were selected and placed onto NGM medium plates (90 mm in diameter) that did not contain *E. coli* OP50. These *C. elegans* were positioned at the center of the medium.

Once the *C. elegans* had slightly crawled away from the edge of the medium, 1 µL of elicitor (0.1% benzaldehyde ethanol solution) was added at a distance of 1 cm from the edge of the plate. As a control, 1 µL of anhydrous ethanol was added to the edge of the medium opposite to the elicitor. Afterwards, 1 µL of 1 M NaN<sub>3</sub> was added to both the elicitor and control points to paralyze any *C. elegans* that approached these areas. After the *C. elegans* had crawled for 2 hours at 20 °C, the number of paralyzed *C. elegans* within a radius of 1 cm around the elicitor and the control was recorded.

$$\text{Convergence Index (CI)} = \frac{\text{number of attracted } C. elegans - \text{number of control } C. elegans}{\text{total number of } C. elegans \times 100\%}$$

**2.2.5. Effect of sucralose on NaCl chemotaxis in the *C. elegans*.** According to the method described by Bargmann et al. [28], a petri plate with a diameter of 90 mm was prepared. On this plate, two points labeled as A and B were selected, positioned 4 cm apart from each other. The NGM medium used at these points did not contain NaCl and *E. coli* OP50. Point A served as the control point, while at point B, a piece of agar containing 100 mM NaCl was placed. The plate was then left overnight at 4 °C. Afterward, the agar at point B was carefully removed, resulting in the formation of a continuous NaCl concentration gradient extending from point B towards point A. Point C was selected on the center vertical line between points A and B. The distance between point C and both points A and B was 3 cm. Point C served as the starting point for subsequent procedures.

*C. elegans* synchronized to the L1 stage were transferred to the NGM medium of each group and incubated at 20 °C for 48 hours (Table 1). After that, the groups were washed with NaCl-free M9 buffer.

**Table 1.** Treatment of Different Groups

Group	Treatment	Sample size
Standard NGM	No hunger at 20 °C	50
NGM without NaCl and OP50	Starvation at 20 °C for 4 h	50
NGM of 100 mM NaCl without OP50	Starvation at 20 °C for 4 h	50

At the end of the treatment period, the *C. elegans* were dropped onto the starting point C on the petri plate. Any excess solution was carefully removed using paper. Once the *C. elegans* had crawled away from the starting point C, 1 µL of 1 M NaN<sub>3</sub> was added to both points A and B on the petri plate. The *C. elegans* were then allowed to freely move for 2 hours at a temperature of 20 °C. At the end of this period, the number of *C. elegans* present within a radius of 1 cm around points A and B were counted separately.

$$\text{Convergence Index (CI)} = \frac{\text{number of } C. \text{ elegans in point B area} - \text{number of } C. \text{ elegans in point A area}}{\text{total number of } C. \text{ elegans} \times 100\%}$$

**2.2.6. Effect of sucralose on the related gene expression in the *C. elegans*.** RNA Extraction. *C. elegans* eggs, with a minimum of 50 eggs per group, were cultured using NGM medium supplemented with different concentrations of sucralose. The control group was cultured using standard NGM medium (in 60 mm plates). For each group, 2000 clean D1 *C. elegans* were carefully picked and transferred to separate 1.5 mL EP tubes. The *C. elegans* were then washed three times using H<sub>2</sub>O. Next, RNase-free H<sub>2</sub>O was added to the tubes, and the *C. elegans* were transferred to RNase-free centrifugation tubes. The tubes were centrifuged to remove the supernatant. Subsequently, the TRUEScript RT MasterMix kit was used to prepare for PCR. 50 µL of lysate was added to each centrifugation tube containing the *C. elegans*. 20 µL of the sample was then transferred to RNase-free PCR tubes. The PCR tubes were then incubated at 65°C for 10 minutes in a PCR machine. After incubation, the samples were inactivated at 85 °C for 1 minute and cooled on ice. The RNA concentration and quality were determined to ensure the suitability of the extracted RNA for further analysis.

*cDNA reverse transcription.* 2 mg of total RNA was taken and first-strand cDNA synthesis was performed using the TransScript First-Strand cDNA Synthesis kit.

*RT-qPCR.* The expression levels of *age-1*, *add-1*, *flp-34*, *dop-1*, *tph-1*, *eat-4*, *glr-1*, *nmr-1*, *nmr-2*, *casy-1* and *unc-43* in each group of samples were detected by using SYBR Green PCR Master Mix with *gapdh* as an internal reference (IR). The sequences of PCR primers used in the experiment are shown in Table 2. The relative expression of target genes was analyzed by the 2<sup>-ΔΔCt</sup> method.

**Table 2.** RT-qPCR primers sequences.

No.	Primer	Sequence (5' to 3' direction)
IR	<i>gapdh</i> -F	GCTGACGGACCAATGAAG
	<i>gapdh</i> -R	TGACGAAGTGTGGGTTGA
1	<i>age-1</i> -F	CGTTCGGAAGTGGAAAGCTATCG
	<i>age-1</i> -R	GAGTACTGCAGATGGTGGCATATC
2	<i>add-1</i> -F	GTTTCATGACGTCAACGTTCCATCC
	<i>add-1</i> -R	GGATTCGGCGCATAGATTTGGTG
3	<i>flp-34</i> -F	GCAGACATTTCCACATTTGCATCAG
	<i>flp-34</i> -R	GTACTGATCTTCCGATGATGGAATGATG

**Table 2.** (continued).

4	<i>dop-1-F</i>	GCTATTTGCTGCAGTCAACGATATC
	<i>dop-1-R</i>	CTTCCAATTGCATACGGAAGCGG
5	<i>tph-1-F</i>	CATGGCTCTATTCGCTGATCCAG
	<i>tph-1-R</i>	GACATTCTTGCTCAACAACACGATCC
6	<i>eat-4-F</i>	GCAAGAAGAAGGAAACGAAAACCCG
	<i>eat-4-R</i>	GCCCTTGAGTAATTTGAATGAAAGCC
7	<i>glr-1-F</i>	GGTGGAGATGATGTTAGTGTTGAGG
	<i>glr-1-R</i>	CACCTTGTCGCCACGCTAATAC
8	<i>nmr-1-F</i>	GTTCAACGTTACATTGAGGTAGAGCTG
	<i>nmr-1-R</i>	GAAGGGAATTCCATTGAGCATCTACAC
9	<i>nmr-2-F</i>	GTTCCCAAATCTACAGTATCCCGATTG
	<i>nmr-2-R</i>	GTCAAGCACACAGCGTCATAG
10	<i>casy-1-F</i>	CATTCTGGAAATGGACCTCCCG
	<i>casy-1-R</i>	CCGATGACGAGCAACACTAACAG
11	<i>unc-43-F</i>	CAGGATATTGTACGGGTGACTCAGAC
	<i>unc-43-R</i>	GCCTTCGATAAGGTTACCAAGTGC

2.2.7. *Homology analysis.* The NCBI Homologene tool was utilized to determine whether the genes associated with learning and memory in *C. elegans*, which are regulated by sucralose, have homologous counterparts in humans. Homology comparisons were conducted, and the results were summarized for documentation purposes.

2.2.8. *Data processing.* All experiments pertaining to learning and memory were conducted three times to ensure reliability, and the data obtained were expressed as Mean  $\pm$  SEM (standard error of the mean). Statistical analyses were performed to assess the differences between two groups using Student's t-test. For comparisons involving multiple groups, the data were analyzed using the One-way ANOVA test. The statistical analysis was carried out using GraphPad Prism 9.

### 3. Results

#### 3.1. Market research results

A comprehensive field observation was conducted in six small, medium, and large supermarkets to record the presence of various mainstream sugar-substituted beverages. The study focused on beverages from three distinct beverage companies. The observations revealed that all six beverages examined in the study contained sucralose as a sweetener. The details of these findings are summarized in Table 3.

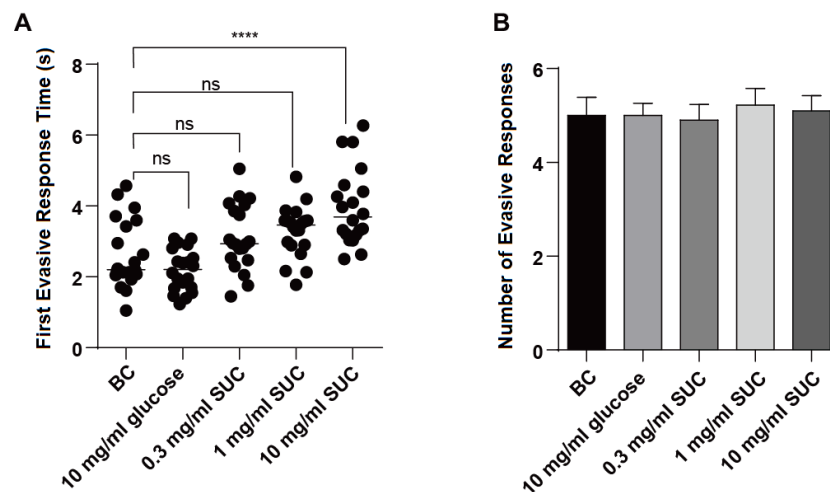
**Table 3.** Results of market research on sugar-substituted beverages.

Manufacturer	Name	Sugar substitutes type	Sugar substitute name	Ingredient list	Contained sucralose
Chi Forest	White Peach Flavored Sparkling Water	Sugar alcohols, synthetic	Erythritol, Sucralose	Water, Erythritol, Carbon dioxide, Sodium bicarbonate, Citric acid, Sucralose, Potassium sorbate, Food flavor.	Yes
Chi Forest	Alien Electrolyte Water	Sugar alcohols, synthetic	Erythritol, Sucralose	Water, Erythritol, Vitamin E, Vitamin B6, Salt, Calcium Lactate, Potassium Chloride, Zinc gluconazole, Citric Acid, Sodium Citrate, Sucralose, Food Flavor	Yes
Coca-Cola	Coca-Cola Zero	Synthetic	Aspartame, Acesulfame, Sucralose	Carbonated Water, Caramel Color, Phosphoric Acid, Aspartame, Potassium Benzoate (To Protect Taste), Natural Flavors, Potassium Citrate, Acesulfame Potassium, Caffeine	Yes
Master Kong	Iced Lemon Black Tea	Sugar alcohols, synthetic	Erythritol, Sucralose, Acesulfame	Water, Polydextrose, Instant Black Tea, Grape Juice Concentrate, Edible Salt, Black Tea Concentrate, Erythritol, Citric Acid, Sodium Citrate, DL-Malic Acid, Sucralose, Sodium D-Isorbate, Vitamin C, Caramel Color, Acesulfame, Edible Flavors	Yes
Coca-Cola	Sprite Zero	Synthetic	Aspartame, Acesulfame, Sucralose	Carbonated Water, Citric Acid, Potassium Citrate, Natural Flavors, Potassium Benzoate (To Protect Taste), Aspartame, Acesulfame Potassium	Yes
Coca-Cola	Fanta Zero	Synthetic	Aspartame, Acesulfame, Sucralose	Carbonated Water, Citric Acid, Potassium Citrate, Aspartame, Natural Flavors, Modified Food Starch, Potassium Benzoate, Acesulfame Potassium, Glycerol Ester Of Rosin, Yellow 6, Medium Chain Triglycerides, Sucrose Acetate Isobutyrate, Red 40	Yes

### 3.2. Effect of sucralose on tap stimulus in the *C. elegans*

The comparison of experimental data on the first evasive response time of *C. elegans* revealed the following results. There was no statistically significant difference in the first evasive response time between the blank control group and the groups treated with 0.3 mg/mL sucralose, 1 mg/mL sucralose, and 10 mg/mL glucose. However, the group treated with 10 mg/mL sucralose exhibited a significant prolongation of the evasive response time compared to the blank control group (\*\*\*\*  $P < 0.0001$ ), and this difference was statistically significant (Fig. 2A). These findings indicate that a high concentration of sucralose significantly increased the evasive response time, reduced the sensitivity of the nematodes, and affected their non-associative learning ability.

Regarding the number of evasive response times of *C. elegans* (Fig. 2B), the experimental data showed no statistically significant difference between the blank control group and all the experimental groups. Therefore, sucralose did not have an impact on the number of evasive responses in *C. elegans*.

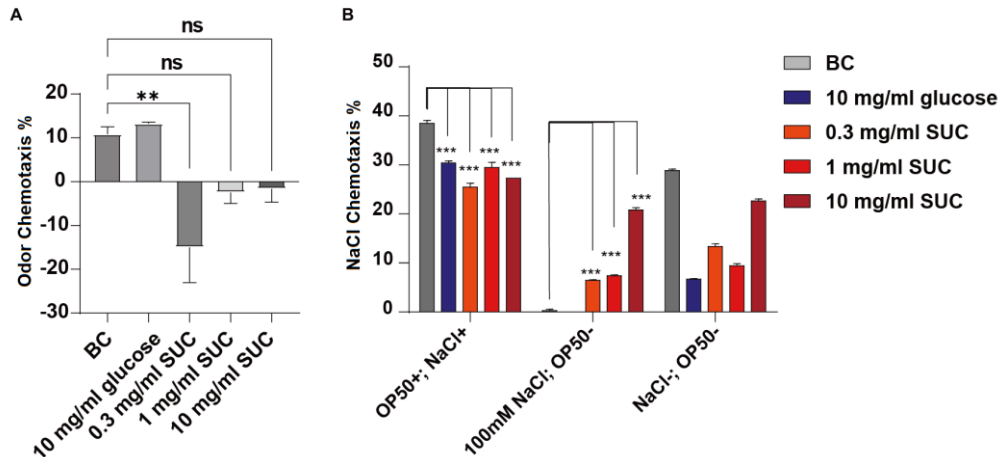


**Figure 2.** Effect of different concentrations of sucralose on tap stimulus of *C. elegans*.

### 3.3. Effect of sucralose on odor chemotaxis in the *C. elegans*

The comparison of experimental data on the percentage of benzaldehyde in *C. elegans* odor chemotaxis (Fig. 3A, \*\*  $P < 0.01$ , ns for no statistical difference) revealed the following findings. There was no statistically significant difference in odor chemotaxis between the blank control group and the groups treated with 1 mg/mL sucralose, 10 mg/mL sucralose, and 10 mg/mL glucose. However, the odor chemotaxis of *C. elegans* in the 0.3 mg/mL sucralose group was significantly lower compared to that of the blank control group, and this difference was statistically significant. These results indicate that the low concentration of sucralose had an impact on the odor chemotaxis of *C. elegans* towards the odor of benzaldehyde.





**Figure 3.** Effect of different concentrations of sucralose on odor chemotaxis and NaCl chemotaxis of *C. elegans*.

### 3.4. Effect of sucralose on NaCl chemotaxis in the *C. elegans*

To investigate the NaCl chemotaxis of *C. elegans*, they were transferred to NGM medium with a high concentration of NaCl and NaCl-free NGM medium, while the standard NGM medium served as the control. After a 4-hour starvation treatment, the *C. elegans* were transferred to chemotaxis plates, and their chemotaxis behavior was assessed.

On the standard NGM plates, the chemotaxis of *C. elegans* towards NaCl was significantly reduced in the treatment groups with 0.3 mg/mL sucralose, 1 mg/mL sucralose, 10 mg/mL sucralose, and the 10 mg/mL glucose control group, as compared to the blank control group (Fig. 3B, \*\*\*  $P < 0.001$ ). These results suggest that sucralose treatment decreased the natural chemotaxis of *C. elegans* towards NaCl.

In the experimental group that combined starvation and a high concentration of 100 mM NaCl, the chemotaxis towards NaCl was significantly reduced in both the blank control group and the 10 mg/mL glucose group. Moreover, all three concentrations of sucralose showed statistical differences compared to the blank control group. This indicates that the addition of sucralose impaired the ability of *C. elegans* to effectively associate the high concentration of NaCl and starvation. These findings suggest that sucralose treatment negatively affected the associative learning ability of *C. elegans*.

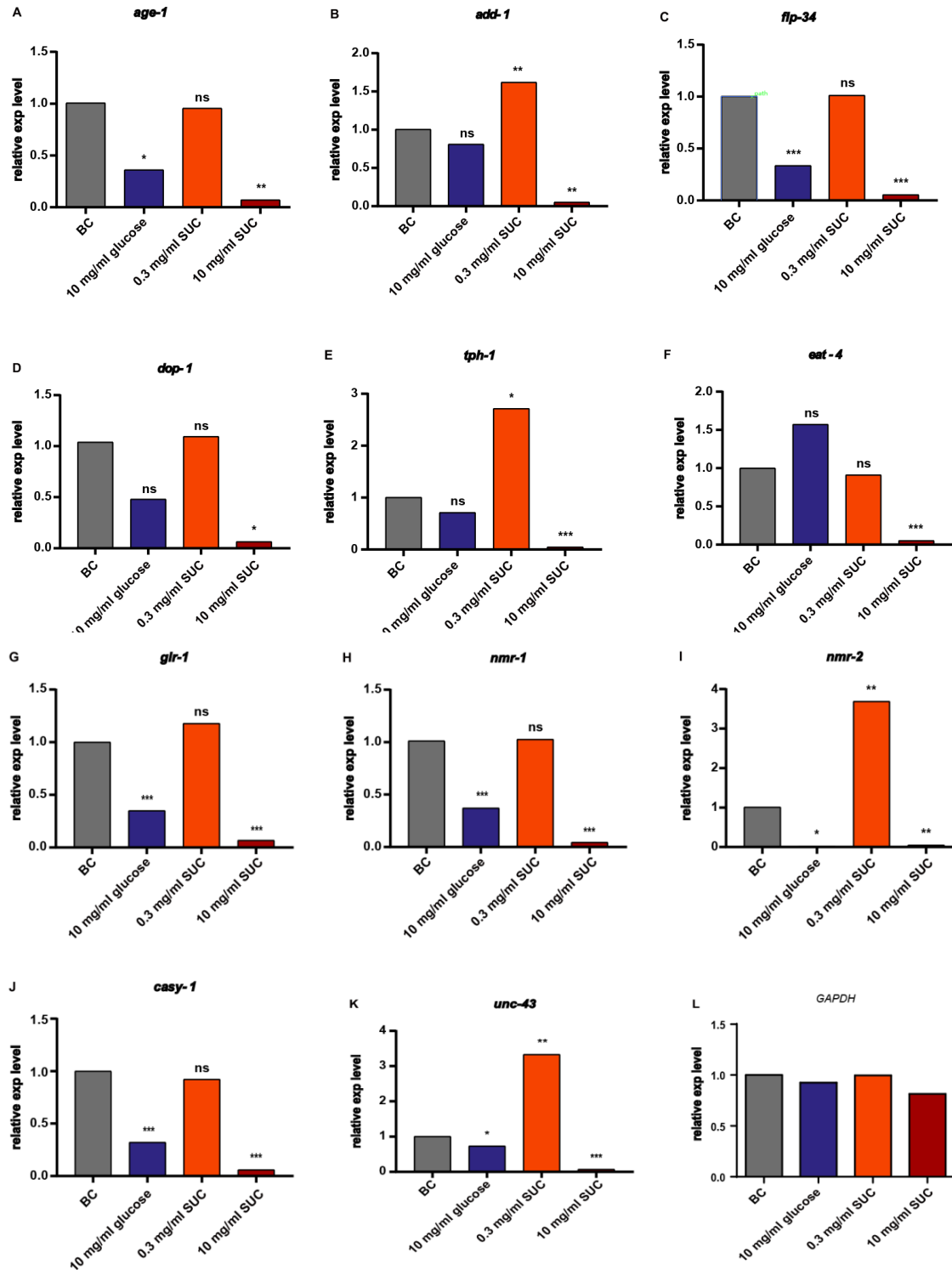
Percentage of NaCl chemotaxis

$$= \frac{\text{number of } C. \text{ elegans at NaCl} - \text{number of } C. \text{ elegans at blank control}}{\text{total number of } C. \text{ elegans}} \times 100\%$$

### 3.5. Effect of sucralose on the related gene expression in the *C. elegans*

In order to explore the mechanism of changes in *C. elegans* learning and memory function induced by different concentrations of sucralose treatment, RT-PCR was used to quantify the relative expression of 11 genes known to be associated with *C. elegans* learning and memory (Fig. 4). These genes include the lifespan-associated PI3K gene *age-1*, the microtubule-binding protein related gene *add-1*, the neuropeptide related gene *flp-34*, the dopamine receptor related gene *dop-1*, the serotonin synthesis enzyme related gene *tph-1*, the calcium-binding protein related gene *casy-1*, and the low-glutamate receptor-related signaling molecules related gene *eat-4*, *glr-1*, *nmr-1*, *nmr-2*, and *unc-43*.

The results demonstrated that the relative expression of *add-1*, *tph-1*, *nmr-2*, and *unc-43* was significantly up-regulated in the low concentration (0.3 mg/ml) sucralose group. Conversely, in the high concentration (10 mg/ml) sucralose group, the relative expression of all 11 genes was significantly down-regulated to less than 0.1-fold of the blank control group, and these differences were statistically significant. These findings indicate that the effect of sucralose treatment on the learning and memory ability of *C. elegans* is achieved through extensive regulation of learning and memory-related pathways.



**Figure 4.** Analysis of the effect of different concentrations of sucralose on the relative expression of genes. *age-1* (A), *add-1* (B), *flp-34* (C), *dop-1* (D), *tph-1* (E), *eat-4* (F), *glr-1* (G), *nmr-1* (H), *nmr-2* (I), *casy-1* (J), *unc-43* (K), all of the above genes were expressed by using *GAPDH* (L) as an internal reference, and the relative expression of all the above genes with blank control group (BC) as a baseline.  
\*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

### 3.6. Homology analysis of sucralose-regulated genes associated with learning and memory in *C. elegans* and human genes

In this study, we used the NCBI-Homologene tool to find out whether the 11 learning memory-related genes regulated by sucralose had homologous genes in humans and the percentage of homology. We found that *nmr-2*, *age-1*, *add-1*, *dop-1*, *tph-1*, *eat-4*, *glr-1*, *nmr-1*, *casy-1* and *unc-43* are all homologous in humans, with homology ranging from 30% to 60% (Table 4). This suggests that these sucralose-regulated genes in *C. elegans* may be similarly affected by sucralose in humans.

**Table 4.** Homology analysis of *C. elegans* and human genes.

Gene_ <i>C. elegans</i>	Gene_ <i>H. sapiens</i>	Homology	Similarities
<i>nmr-2</i>	GRIN2D	yes	36%
<i>age-1</i>	PIK3CA	yes	30%
<i>add-1</i>	ADD1	yes	37%
<i>flp-34</i>		no	
<i>dop-1</i>	ADRA2B	yes	42%
<i>tph-1</i>	TPH2	yes	54%
<i>eat-4</i>	SLC17A7	yes	54%
<i>glr-1</i>	GRIA3	yes	38%
<i>nmr-1</i>	GRIN1	yes	33%
<i>casy-1</i>	CLSTN1	yes	30%
<i>unc-43</i>	CAMK2D	yes	60%

## 4. Discussion

### 4.1. Effects of different concentration of sucralose on the learning memory function of *C. elegans*

To better align with the types of sugar substitutes commonly used in sugar-substituted foods available in the market, the study initially examined six popular sugar-substituted beverages from three companies. Through field observations conducted in the supermarkets in Beijing, it was identified that these beverages utilized sucralose as a sweetener to varying degrees. Based on this observation, the study chose sucralose in combination with the biological model of *C. elegans* to explore the effects of sugar substitutes on learning and memory functions and their mechanisms of action.

In this study, we initially tested the effect of sucralose on the evasive response of *C. elegans* using a tap stimulus assay. The results showed that a high concentration of sucralose significantly prolonged the time of evasive response. This indicates that high concentrations of sucralose treatment can reduce the sensitivity of *C. elegans* to tap stimulus, ultimately leading to a significant reduction in the mechanosensory-related non-associative learning ability of *C. elegans*. The results of the chemotaxis assays, including the benzaldehyde odor chemotaxis experiment and NaCl chemotaxis ability, revealed that low concentrations of sucralose inhibited odor chemotaxis in *C. elegans*. Furthermore, all three concentrations of sucralose treatments inhibited NaCl chemotaxis, suggesting that sucralose treatments significantly reduced the *C. elegans*' innate chemotaxis response to the inducer. Additionally, the NaCl chemotaxis experiments and starvation tests demonstrated that different concentrations of sucralose treatments impaired the associative learning ability of *C. elegans*. This finding indicates that the addition of sucralose reduced the associative learning ability of *C. elegans*.

Combined neuropsychological testing and EEG/qEEG analysis in humans revealed that long-term supplementation with sucralose significantly reduced overall memory, encoding memory, and executive function. This suggests that prolonged consumption of sucralose has an adverse effect on learning and memory functions [29]. Oytun et al. conducted a study on rats, treating them with various types of

artificial sweeteners including sucralose over an extended period. Their findings indicated that long-term consumption of artificial sweeteners impaired cognitive ability and hippocampal integrity [30]. In previous studies, the concentration of sugar substitutes has also been explored to some extent. Jiao et al. investigated the effects of different concentrations of sucralose on various aspects of *C. elegans*, such as lifespan, egg-laying, swallowing, and locomotion. They found that sucralose extended the lifespan of *C. elegans* in a dose-dependent manner, did not affect egg-laying or swallowing frequency, and had a positive effect on locomotion at low concentrations. However, high concentrations of sucralose had adverse effects on the locomotion of *C. elegans* [31]. Another study examined the effect of sucralose on *C. elegans* lifespan and locomotor activity. The findings showed that low concentrations of sucralose (0.03-0.3 mg/mL) significantly extended nematode lifespan and improved locomotor activity. However, high concentrations of sucralose (10 mg/mL) had a tendency to shorten nematode lifespan, although the effect was not statistically significant [32]. These studies suggest a dose-dependent effect of sucralose on various aspects of *C. elegans*' life activities. In this study, we observed differences in the effects of different concentrations of sucralose on learning and memory. For instance, the effects of high and low concentrations of sucralose on non-associative learning behaviors differed.

#### 4.2. The inhibition of learning and memory functions in *C. elegans* by sucralose may be mediated through mechanisms such as glutamate receptor-related signaling pathways

In terms of mechanisms, the current research has not definitively established the specific mechanisms underlying the impairment of learning and memory functions caused by sugar substitutes. However, some studies have suggested that the damage to dopamine neurons may play a role in this impairment [33-35]. Furthermore, it has been reported that the effect of a high carbohydrate diet on the learning ability of *C. elegans* is regulated by different gene networks [36]

To investigate the genetic mechanism underlying the regulation of *C. elegans* learning memory by sucralose, this study employed RT-qPCR technology to analyze 11 genes known to be associated with *C. elegans* learning memory. The goal was to explore how different concentrations of sucralose treatment affect the learning memory function of *C. elegans* and elucidate the underlying mechanisms involved. The genes included *age-1* (PI3K gene associated with lifespan), *add-1* (microtubule-binding protein), *flp-34* (neuropeptide), *dop-1* (dopamine receptor), *tph-1* (serotonin synthesis enzyme), *casy-1* (calcium-synthesizing protein), as well as glutamate receptor-related signaling molecules *eat-4*, *glr-1*, *nmr-1*, *nmr-2*, and *unc-43*. The results revealed significant up-regulation of *add-1*, *tph-1*, *nmr-2*, and *unc-43* gene expressions in the low concentration (0.3 mg/mL) sucralose treatment group. Notably, *nmr-2* showed a 3.69-fold up-regulation compared to the blank control group. In contrast, the high concentration (10 mg/mL) sucralose treatment group exhibited significant down-regulation of all 11 genes to less than 0.1-fold compared to the blank control group. These differences were statistically significant. These findings suggest that sucralose treatment affects the learning memory ability of *C. elegans* through extensive regulation of pathways associated with learning and memory.

Among the 11 genes analyzed, the changes observed in glutamatergic neuron-related genes were more prominent compared to the dopamine neuron-related gene *dop-1*. Specifically, the expression levels of genes such as *nmr-1/2*, which encode NMDA-type glutamate receptor subunits, were significantly decreased in the high concentration sucralose group. This suggests that high concentrations of sucralose may lead to impaired non-associated as well as associated learning and memory functions in *C. elegans* by damaging glutamatergic neurons. Interestingly, low concentrations of sucralose were found to promote the expression of certain relevant genes. This finding may seem contradictory to the impaired odor chemotaxis observed in *C. elegans*. However, it is possible that the increased expression of *add-1* promotes the expression of *glr-1*, resulting in an excessive increase in the content of the AMPA-type glutamate receptor [37]. This, in turn, raises the threshold of glutamate signaling levels required to activate the receptor, ultimately impairing the learning memory function of *C. elegans*.

There are existing findings regarding the regulation of learning memory by the remaining genes in question (wormbase.org). For instance, the lifespan-related gene *age-1* has been shown to influence intracellular signaling and metabolic regulation by modulating the activity of the insulin/PI3K signaling

pathway, which in turn affects learning behavior. Mutations in *tph-1*, a gene responsible for serotonin synthesis, have been found to impact the synthesis of 5-HT (serotonin) in *C. elegans*, thereby affecting their ability to learn odor-food associations. The gene *unc-43*, which encodes a Ca<sup>2+</sup>/Calmodulin-dependent protein kinase, has been implicated in PA14 aversive learning. In the present study, the regulation of these pathways by sucralose was investigated, and it was observed that gene expression was significantly down-regulated, especially when high concentrations of sucralose were applied.

#### 4.3. Effect of sucralose on human learning memory function and its mechanism

Human studies have demonstrated that long-term consumption of sucralose can lead to significant decreases in overall memory, encoded memory, and executive functioning [29]. Long-term or excessive consumption of sugar substitutes may contribute to metabolic disorders, increased nighttime sweet intake, and a decline in learning and memory capacity. [38, 39]. Animal experiments with immature rats have demonstrated that the long-term habitual intake of low-calorie sweeteners can have lasting effects on glucose regulation, sugar-driven behaviors, and memory processes that rely on the hippocampus. These effects may be attributed, at least in part, to changes in the expression of nutrient transporters, sweet taste receptors, and central gene pathways [40]

In this study, it was observed that sucralose can impact the learning and memory capacity of *C. elegans* by broadly regulating a network of genes associated with learning and memory. Several of the learning and memory-related genes affected by sucralose, such as *nmr-2*, *age-1*, *add-1*, *dop-1*, *tph-1*, *eat-4*, *glr-1*, *nmr-1*, *casy-1*, and *unc-43*, have homologous counterparts in humans. This finding lays the groundwork for applying the research findings to explore the effects and mechanisms of sugar substitute consumption in humans. For example, the mouse homolog of *nmr-2*, *Grin2d*, has been identified as a subunit of the NMDA receptor in hippocampal interneurons, playing a role in excitatory synaptic transmission [41]. The human homolog of the *dop-1* gene in *C. elegans*, known as ADRA2B, has been associated with certain cognitive effects when mutated in humans. Male carriers of this gene mutation exhibit impaired recognition memory under stress conditions, while female carriers show enhanced long-term memory, increased emotional memory, greater amygdala response to emotional stimuli, and increased intrusion of trauma memories, making them more susceptible to post-traumatic stress disorder [42, 43]. Therefore, the high concentration of sucralose proposed in this study, based on the effects observed in *C. elegans*, may potentially impair their non-associative and associative learning and memory abilities by causing damage to glutamatergic neurons. This finding may also provide insights into the safety and mechanisms of sugar substitute consumption in humans, given the high genetic homology between *C. elegans* and humans.

#### 4.4. Potential neurodevelopmental harms of sugar-substituted beverages in the adolescent population

Sugar-substituted beverages have gained popularity as an alternative to sugar-sweetened beverages, particularly among individuals who are health-conscious or seeking to reduce their sugar intake. According to a report from the Centers for Disease Control and Prevention (CDC), approximately one-fifth of the U.S. population consumed sugar-substituted beverages on a daily basis during the period of 2009-2010. The percentage of women consuming these beverages increased from 17.8% to 21.2%, while for men, it increased from 13.9% to 19.0% between 1999-2000 and 2009-2010. The growing influence of sugar-substituted beverage advertising has contributed to the increasing market share of these beverages [10]. However, research indicates that only women, the elderly, and individuals with disabilities possess some knowledge about sugar substitutes, while young people have limited awareness [44]. Despite the numerous benefits of sugar substitutes compared to caloric sweeteners, such as their potential contributions to weight control, blood glucose management, reduced dental problems, and expanded beverage choices, this study uncovers a concerning finding. It highlights that the consumption of sugar substitutes can have adverse effects on learning and memory functions, particularly when used in excessive amounts. These findings raise concerns about the potential hazards of sugar substitutes for adolescent neurodevelopment.

## 5. Conclusion

In this study, the developmental stage of *C. elegans* was chosen as the experimental model to investigate the impact of three different concentrations of sucralose on non-associative and associative learning abilities. In the non-associative learning experiment, *C. elegans* treated with high concentrations of sucralose exhibited a significant prolongation in withdrawal reaction time, while those treated with low concentrations of sucralose showed a significant reduction in odor chemotaxis. In the associative learning experiment, different concentrations of sucralose adversely affected the associative learning ability of *C. elegans*. To understand the underlying regulatory mechanisms, gene expression changes of 11 *C. elegans* genes associated with learning and memory were analyzed using RT-qPCR after sucralose treatment. The results showed that low concentrations of sucralose significantly up-regulated the relative expression of four gene groups, namely *add-1*, *tph-1*, *nmr-2*, and *unc-43*, while high concentrations of sucralose led to a significant down-regulation of the relative expression of all the genes, particularly the glutamate receptor signaling pathway represented by *nmr-2*.

Overall, the study suggests that high concentrations of sucralose can diminish the learning and memory abilities of *C. elegans* by extensively modulating pathways related to learning and memory. Since homology analysis revealed that 10 out of the 11 genes have counterparts in humans, it is plausible that human consumption of sucralose may also impact the expression levels of these learning and memory-related genes. Consequently, the study highlights the potential harm of sucralose intake in the adolescent population and provides a theoretical foundation for further investigating the effects of sucralose on learning and memory in both mammalian experimental animals and humans.

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