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β-Tubulin mRNA Expression Profiles During Long-Term Memory Formation in Classically Conditioned C. Elegans

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Summary

We report on the mRNA expression profiles of β-tubulin genes in Caenorhabditis elegans during the process of classical conditioning, a type of associative learning. In this research, based on the model that suggests the formation of new synaptic connections for long-term memory storage by means of cytoskeletal rearrangement, we measure the expressional changes on the β-tubulin mRNA levels via quantitative real-time PCR. Using this method, we find that β-tubulin mRNA levels do not significantly change in C. elegans during classical conditioning and long-term memory formation process.

Key words: C. elegans, learning, memory, long-term memory, classical conditioning, tubulin

Klasik Koşullanmış C. Elegans'ta Uzun-Dönemli Bellek Oluşumu Sırasında β-Tübülin mRNA İfadelenme Profilleri

Özet

Bu araştırmada, Caenorhabditis elegans'taki β-tubulin genlerinin, ilişkilendirmeli (assosiyatif) öğrenmenin bir türü olan klasik koşullanma süreci sırasındaki mRNA ifadelenme profillerini sunuyoruz. Uzun dönem bellek oluşumu için yeni bir sinaptik bağlantı oluşturulduğunu ve bu esnada hücre iskeletinin yeniden düzenlendiğini öneren model göz önüne alınmış ve bu modeli test etmek amacıyla farklı β-tübülin genlerinin mRNA seviyeleri nicel Gerçek-Zamanlı PCR yöntemiyle ölçülmüştür. Sonuç olarak, C. elegans'ta, klasik koşullanma ve uzun dönem bellek oluşumu esnasında β-tubulin mRNA seviyelerinin anlamlı olarak değişmediği bulunmuştur.

Anahtar Kelimeler: C. elegans, öğrenme, bellek, uzun-dönemli bellek, klasik koşullanma, tübülin

INTRODUCTION

Researches on the cellular basis of experience dependent behaviors (or learning) and memory have revealed several phases of memory that can be distinguished by their permanence. It was shown that these memory phases include some forms of intermediate-term memories in addition to traditional long-term and short-term memory distinction[24,25].

It was previously proposed that the mechanism of the memory storage initially occurs as short-term memory and consequently it is consolidated as long-term memory. According to this concept, short-term and long-term memory formations are serial processes. In contrast to this widely accepted notion, recent researches support that these two types of memory formation phenomena are probably parallel processes[12].

Considering the training process of the organism during classical conditioning experiments, main differences between the formation of long-term and short-term memory are related with the differences in number of trials and in intertrial intervals. Studies have shown that a distinct level of increase in number of trials and in intertrial intervals is necessary for long-term memory formation[21] In addition to this, it has been shown that the formation of long-term memory requires mRNA and protein synthesis, unlike formation of short-term memory[6,21,9,16,23]. Studies on sensitization, which is a type of non-associative learning, in the sea slug Aplysia californica have revealed that long-term memory formation requires new synaptic connections between neurons[2,3,4], unlike short-term memory formation.

C. elegans is capable of migrating to attractant chemicals and avoiding repellant chemicals[11,26], and is capable of performing both non-associative[7,20,28] and associative[28,17,18,13] learning.

In this study, with a repellent chemical (acetic acid), the natural chemotaxis behavior of C. elegans is altered via associative learning; and by means of repeated trials, this change leads to formation of long-term memory in the organism. Here we report that, β-tubulin mRNA expression levels, which might be an indicator of cytoskeletal rearrangement mediated formation of new synaptic connection, does not significantly change in C. elegans during long-term memory formation process.

MATERIAL AND METHODS

Cultivation

Bacteria Culture:

E. coli, strain Ade- (The culture collection of Hıfzısıhha) is used for nematode culture. Cultivation of E. coli is made as suggested by Theresa Stiernagle, in 1999 (Stiernagle, 1999)[31].

Nematode Culture:

C. elegans, strain N2 (Bristol) is obtained from Dr. David de Pomerai who is a researcher at Nottingham University. 2.5µL/mL Penicillin-Streptomycin-Neomycin (Biological Industries) combination and 20unit/mL Nystatin dihydrate (Applichem) are added to NGM medium prepared according to the Stiernagle (Stiernagle, 1999)[31]. 100µL of E. coli stock combination is added to each 60mm NGM plate. Nematodes are grown at temperatures between 20-25^{o}C.

Population Chemotaxis Assays

The population chemotaxis assay (P.C.A.) is performed as described by Bargman et al. in 1993[5]. Assay plates are 95mm Petri plates containing; % 1.6 agar, 5mM potassium phosphate (pH= 6), 1 mM. CaCl2 and 1 mM. $MgSO_4$. Two marks are pointed at the opposite sides on the bottom of the plates (approx. 5mm away from the periphery of the plate); the one where attractant chemical was dripped is named as “attractant end” and the other mark is named as “opposite end”. During the preparation step for P.C.A., 60mm plates containing well-fed animals are washed with 2mL S basal solution[8] into 15mL centrifuge tube. After wash, the tube is centrifuged for one minute (at 1500 rpm). The pellet is washed with 5mL dH_20 and the same centrifugation is performed again and consequently the nematodes are cleared of bacteria. These bacteria-free nematodes are put into 1.5mL microcentrifuge tubes and are centrifuged for one minute (at 100 rpm). At the end of this procedure, the adult nematodes are palletized; the larvae floating in the liquid part are thrown away. This step is duplicated and plastic Pasteur pipettes are used to prevent nematodes from getting hurt during these preparation steps.

100-150 of the adult nematodes, which were prepared for the P.C.A. are placed at the center of the plates, and gently dried with a napkin. Subsequently, 1µL of 1:100000 diacetyl (Merck), a volatile chemical that is attractant for C. elegans, is

dripped on to the “attractant end”; the lid of the plate is closed immediately after. Since the nematodes do not stay at the attractant source, in all experiments the nematodes are anesthetized when they reach the attractant end. To achieve this, sodium azide (1M, 1.5µL) is added on both to the “attractant end” and to the “opposite end” before the experiments.

An example of a P.C.A. is shown in Figure 1. The assay is initiated just after closing the lid of the plate and finished 60 minutes later. Subsequently the nematodes at the “attractant end” and at the “opposite end” are counted under a microscope. The total number of nematodes in the assay is also determined and a specific chemotaxis index (C.I.) is calculated as: C.I. = (Number of nematodes at the "attractend end") – (Number of nematodes at the "opposite end")/(Total number of nematodes used in the assay)

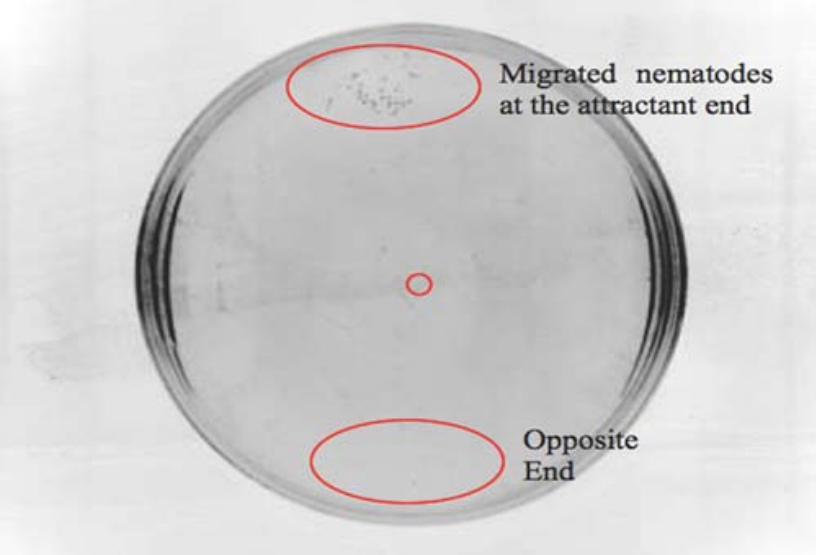

Figure 1*: An example of a Population Chemotaxis Assay. The plate shows the nematodes migrated towards diacetyl, after 1 hour.*

Population Olfactory Conditioning Procedure

Population olfactory conditioning experiments (P.O.C.E.) are performed as described by Morrison et al.[17]. The well-fed adult animals that are used in P.O.C.E. are first washed and then cleared of larvae as described in preparation steps for P.C.A. The nematodes prepared for P.O.C.E. are put into a metal sieve with a #400 mesh screen (height: 5mm , length: 20mm , width: 20mm; Akyol Ticaret, Ulus, ANKARA), which was previously washed with 1:10 Tween 20. After the nematodes are slightly dried with a napkin, the conditioning experiment is immediately initiated.

In all of the experiments, the conditioning stimulus (CS) is the vapor of 6.5 ml of 1:100,000 DA in water. The unconditioned stimulus (US) is 25 ml of 10% acetic acid. Conditioning and testing procedures are performed as previously described[17]. Classical conditioning is performed as the following: By placing the sieve that contains the nematodes, directly atop the vapor of the diacetyl solution, nematodes are exposed to CS for 10s; the critical point here is that making of that the sieve does not touch the liquid (thus nematodes are only exposed to the diacetyl vapor and not

the diacetyl liquid). Immediately after 10s of exposure to the CS, the nematodes are immersed in acetic acid solution for 3s. A single CS exposure plus a single US exposure constitute one single trial, together. Each entire conditioning experiment consists of six trials and every single trial of this six are separated from each other by a 1-minute intertrial interval. At the beginning of each intertrial interval (immediately after the US exposure), the nematodes are given a brief wash with approximately 40 ml of dH_2O for 10 s. After the sixth trial, the nematodes received a final wash with dH_2O and then rinsed from the sieve directly into a 1.5mL conical centrifuge tube, and immediately afterwards, skipping the preparation steps, the P.C.A. is performed.

CS-only Conditioning

Conditioning with the CS only, as a control for unconditioned effects of diacetyl exposure, is performed as described above, with the following exception: After each 10s of exposure to diacetyl vapor, the nematodes are immersed in dH_2O instead of acetic acid during conditioning. All other aspects of conditioning, testing and scoring are exactly as described above.

US-only Conditioning

Conditioning with the US only, as a control for unconditioned effects of acetic acid exposure, is performed as described above, except that the 10s exposure to diacetyl vapor is replaced with a 10s exposure to dH_2O vapor. Steps after this procedure are same with the steps after P.O.C.E.

Long-term (24-hour) Memory

Following P.O.C. experiment, a group of nematodes are transferred to NGM plates, seeded with E. coli and placed at their regular cultivation places for 24 hours. At the end of this time, P.C.A. are performed on the animals in order to test the long-term memory formation.

RNA Isolation and Single Strand cDNA Synthesis

At the end of P.O.C.E., RNA is isolated from the whole population, using total RNA isolation kit (Promega, SV Total RNA Isolation Kit). RNA is isolated at the end of 30 minutes after the conditioning process. It is considered that the first 1-hour-period after training is the critical time period for the protein synthesis required for long-term memory formation. If the cDNA is not synthesized immediately, RNA is kept at -80^{o}C.

Isolated RNA is measured at spectrophotometer (nanodrop). cDNA is synthesized as described in M-MLV Reverse Transcriptase (Promega) usage guidepaper: First, into a 200µL PCR tube; 400ng of the RNA sample and 200ng of the oligo dT are added in a total volume of 15µL in %1 DEPC. After the mixture is heated in a thermal cycler for 5 minutes, the tube is cooled down immediately on ice, and 1µL of 10mM dNTP mixture, 4µL of M-MLV 5x Reaction buffer, 200 units of M-MLV RT are added. Tubes are heated 42^{o}C for 1 hour in a thermal cycler, afterwards. If the amplified cDNA is not used immediately, kept at -20^{o}C.

Quantitative Real-Time PCR

Quantitative Real-Time PCR is performed as described in Light Cycler DNA Master SYBR Green I kit (Roche). The sequences and lengths of primers used in PCR are shown in Table-1. (Primers are designed at ABI Prism Primer Express software and are checked from nucleotide-nucleotide BLAST database).

Table 1: The sequences and lengths of primers designed and used in RT-PCR.

Genes	Primers	(Tm.)	Amplified length (Base pair)
tbb-1	forward: 5'AACGCTGATCTTCGCAAGTTGG 3' backward: 5' TCAATGCACGGTAAGCCTGA 3'	58 °C	115 b.p.
tbb-2	forward: 5' AATCCGCGAAGAGTACCCAGAC 3' backward: 5' TTCTCAACAAGCTGGTGGACG 3'	57 °C	123 b.p.
tbb-4	forward: 5' TTCATATCCAGGCAGGTCAGTG 3' backward: 5' AGTCTCCATTGTATGCTCCGGT 3'	57 °C	105 b.p.
tbb-6	forward: 5' ATGTTCCTCGTGCGATTTTGG 3' backward: 5' ACTGACCGCATTTTCCGGA 3'	56 °C	101 b.p.
cct-5	forward: 5' TTTCCAGTCGAGAATCGCGA 3' backward: 5' CGACAGCGATTTCTGCGAAT 3'	56 °C	103 b.p.

RESULTS AND DISCUSSION

Chemotaxis Indexes of conditioned and unconditioned animals at 0^{th} hour and 24^{th} hour are compared in Figure-2. There is no significant difference between the chemotaxis indexes of CS-only conditioned, US-only conditioned and naive animals (data not shown).

A non-parametric Kruskal-Wallis statistical analysis ($X^2 = 11.802$; sd within group = 15; sd between groups = 2; $p = 0.003<0.05$) revealed that there is a significant difference between the groups concerned. After a multiple comparison, a significant difference is revealed between these groups: Naive-0^{th} hour, $p = 0.025<0.05$; naive-24^{th} hour, $p = 0.000<0.05$. Comparisons between 0^{th} hour and 24^{th} hour demonstrate no significant difference ($p = 0.926>0.05$).

After performing quantitative Real-Time PCR on β-tubulin cDNA's of naive animals and animals conditioned by different procedures, the value of cycle threshold (cT) of each gene is divided by the value of cycle threshold of the house-keeping gene, cct-5 (a subunit of RNA polymerase II) and the rates on the Table-2 are found.

The ratios on the Table-2 are analyzed by non-parametric Friedman method and the results of $X^2 = 1.800$; sd = 3; $p = 0.615>0.05$ are revealed. These results indicate that in each of these four conditions, same situations occur. Using the rates on the Table-2 again, four different cases are analyzed by parametric t-test; after paired-comparison of the rows, the results on Table 3 are revealed.

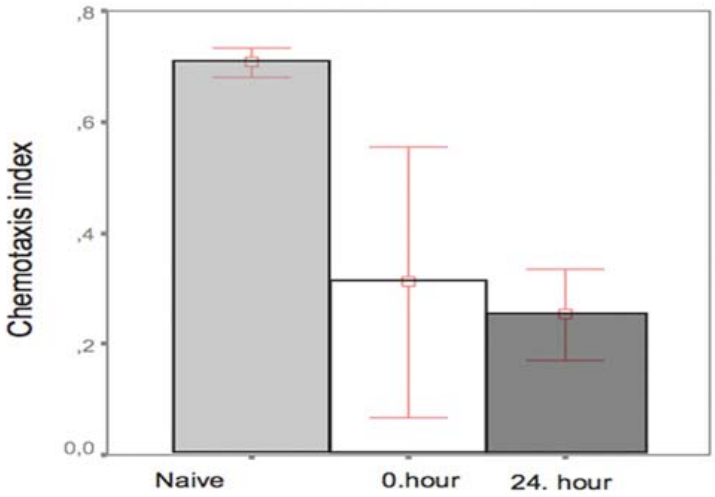

Figure 2: *Comparison of chemotaxis Indexes of conditioned and unconditioned animals at 0th hour and 24th.*

Table 2: Ratios of value of cycle threshold (cT) of each gene divided by the value of cycle threshold of the house-keeping gene.

	tbb-1	***tbb-2***	***tbb-4***	***tbb-6***	***cct – 5*** **cycle threshold values**
Naive (1st case)	0,79	0,87	1,03	1,35	21,11
Classically conditioned (2nd case)	0,88	1,05	0,84	1,25	19,82
CS-Only conditioned (3rd case)	0,83	0,83	0,96	1,38	18,60
US-Only conditioned (4th case)	0,91	0,95	1,06	1,33	20,29

After analysis of the mRNA expression rates of these various β-tubulin genes, which causes the synthesis of one of the main elements of cytoskeletal organization, it is revealed both by non-parametric Friedman method and by parametric t-test that there is no significant difference between the cases in terms of gene expression rates. These results indicate that C. elegans does not undergo a significant cytoskeletal alteration in terms of de novo microtubule synthesis, during classical conditioning based long-term memory formation.

On the other hand, in addition to their critical role in vertebrate synaptic plasticity, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) type of ionotropic glutamate receptors and glutamate neurotransmission has also been shown to be involved in habituation of the tap withdrawal response as well as in olfactory associative learning in Caenorhabditis elegans[21,22,18]. These studies on C. elegans demonstrated that the upregulation of AMPA type glutamate receptors is required for long-term memory formation, independent of the type of learning procedure (either it is habituation, which is a type of non-associative learning or is classical conditioning, which is a simple form of associative learning). Consequently, regarding these researches on learning and memory in C. elegans, a possible mechanism for the long-term memory formation, which is illustrated in Figure 3, can be suggested as follows: glr-1 encodes an AMPA-type ionotropic glutamate receptor subunit and it was first implicated in a learning paradigm by Morrison and van der Kooy[18]. They demonstrated that glr-1 mutant nematodes were impaired in olfactory associative learning, but responded normally to both the conditioned stimulus and the unconditioned stimulus. In C. elegans, AWA is the main neuron that senses diacetyl. ASH has an important role among the sensory neurons that mediate acetic acid aversion. It seems that associative learning requires the upregulation of the AMPA type glutamate receptors GLR-1 in AIB, which is the interneuron between AWA and ASH. This indicates that the long-term memory may be a type of synaptic plasticity based on an elemental form of Long Term Potentiation in Caenorhabditis elegans.

Table 3. Statistical comparison of different cases of conditioning.

Case 1 vs 2: $p = 0,975 > 0,05$
Case 1 vs 3: $p = 0,957 > 0,05$
Case 1 vs 4: $p = 0,748 > 0,05$
Case 2 vs 3: $p = 0,976 > 0,05$
Case 2 vs 4: $p = 0,681 > 0,05$
Case 3 vs 4: $p = 0,711 > 0,05$

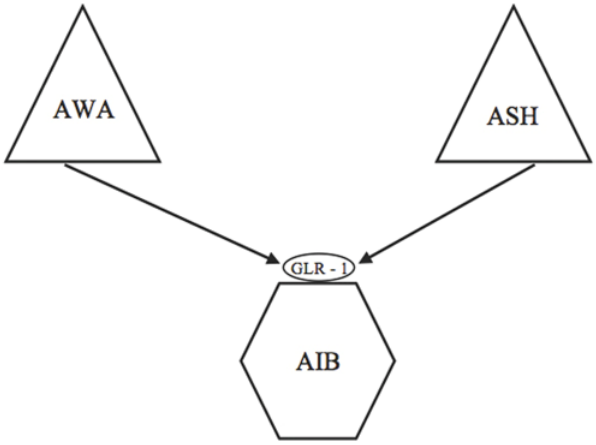

Figure 3: *A schematic for a possible LTP-based long-term memory formation during classical conditioning of diacetyl sensation in C. elegans.*

As a conclusion, further detailed researches altering the trial numbers and intertrial intervals, which may cause different type of memories based on different mechanisms, will contribute to better understanding of the nature of memory in C. elegans.

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REFERENCES

1. *Albert, P. S., Brown S. J., Riddle D. L. Sensory control of dauer larva formation in C. elegans. Journal of Comparative Neurology 1981; 198:435-451.*
2. *Bailey, C. H., and Chen, M. Morphological basis of long-term habituation and sensitization in Aplysia. Science 1983; 220, 91–93.*
3. *Bailey, C. H., and Chen, M. Long-term memory in Aplysia modulates the total number of varicosities of single identified sensory neurons. Proc. Natl. Acad. Sci. USA 1988a; 85, 2373–2377.*
4. *Bailey, C. H., and Chen, M. Long-term sensitization in Aplysia increases the number of presynaptic contacts onto the identified gill motor neuron L7. Proc. Natl. Acad. Sci. USA 1988b; 85, 9356–9359.*
5. *Bargmann C. I., Hartweig E. A., Horvitz H. R. Odorant-selective genes and neurons mediate olfaction in C. elegans. Cell 1993; 74:515-527.*
6. *Beck, C. D. O., & Rankin, C. H. Heat shock disrupts long-term memory consolidation in Caenorhabditis elegans. Learning and Memory 1995; 2, 161-177.*
7. *Beck, C. D. O., & Rankin, C. H. Long-term habituation is produced by distributed training at long ISIs and not by massed training at short ISIs in Caenorhabditis elegans. Animal Learning and Behavior 1997; 25, 446 – 457.*
8. *Brenner, S. The genetics of Caenorhabditis elegans. Genetics 1974; 77, 71 – 94.*
9. *Castellucci V. F. , Blumenfeld H., Goelet P., Kandel E. R. Inhibitor of protein synthesis blocks long-term behavioral sensitization in the isolated gill-withdrawal reflex of Aplysia. J Neurobiol. 1989; 20(1):1–9.*
10. *Chalfie, M. , Sulston, J. Developmental genetics of the mechanosensory neurons of Caenorhabditis elegans. Dev. Biol. 1981; 82: 358 – 70.*
11. *Dusenbery, D. B. Analysis of chemotaxis in the nematode Caenorhabditis elegans by counter current separation. J. Exp. Zool. 1974; 188: 41 – 47.*
12. *Emptage N. J., Carew T. J. Long-term synaptic facilitation in the absence of short-term facilitation in Aplysia neurons. Science 1993; 262(5131): 253 - 256.*

13. *Gomez M., de Castro E., Guarin E., Sasakura H., Kuhara A., Mori I., Bartfai T., Bargmann C. I., Nef P. Ca2+ signaling via the neuronal calcium sensor-1 regulates associative learning and memory in C. elegans. Neuron 2001; 30:241-248.*
14. *Hebb, D. O. The organisation of behavior. John Wiley, New York 1949.*
15. *Hedgecock E. M., Russell, R. L. Normal and mutant thermotaxis in the nematode Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 1975; 72: 4061 – 65.*
16. *Montarolo P. G., Goelet P., Castellucci V. F., Morgan J., Kandel E. R., Schacher S. A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in Aplysia. Science 1986; 234(4781): 1249-54.*
17. *Morrison, G. E., Wen, J. Y. M.; Runciman S., van der Kooy, D. Olfactory associative learning in Caenorhabditis elegans is impaired in lrn-1 and lrn-2 mutants. Behavioral Neuroscience 1999; 113: 358-367.*
18. *Morrison, G. E., and van der Kooy, D. A mutation in the AMPA-type glutamate receptor, glr-1, blocks olfactory associative and nonassociative learning in Caenorhabditis elegans. Behav. Neurosci. 2001; 115, 640–649.*
19. *Perkins L. A., Hedgecock E. M., Thomson J. N., Culotti J. G. Mutant sensory cilia in the nematode C. elegans. Developmental Biology 1986; 117:456-487.*
20. *Rankin, C. H., Beck, C. D. O., & Chiba, C. M. Caneorhabditis elegans: A new model system for the study of learning and memory. Behavioral Brain Research 1990; 37, 89 – 92.*
21. *Rose J. K., Kaun K. R., Rankin C. H. A new group-training procedure for habituation demonstrates that presynaptic glutamate release contributes to long-term memory in Caenorhabditis elegans. Learning & Memory 2002; 9:130-137.*
22. *Rose JK, Kaun KR, Chen SH, Rankin CH (2003) GLR-1, a non-NMDA glutamate receptor homolog, is critical for long-term memory in Caenorhabditis elegans. J Neurosci 23: 9595–9599.*
23. *Sahley, C. & Crow, T. Invertebrate learning: Current perspectives. In J. L. Martinez Jr.&R. P. Kesner (Eds.), Neurobiology of learning and memory (pp. 177 – 209). San Diego, CA: Academic Press 1998.*
24. *Schwartz J. H., Castellucci V. F. , Kandel E. R. Functioning of identified neurons and synapses in abdominal ganglion of Aplysia in absence of protein synthesis. J Neurophysiol 1971; 34: 939-953.*
25. *Sutton M. A., Masters S. E., Bagnall M. W., Carew T. J. Molecular mechanisms underlying a unique intermediate phase of memory in aplysia. Neuron 2001; 31(1):143-54.*
26. *Tully T., Preat T., Boynton S. C., Del Vecchio M. Genetic dissection of consolidated memory in Drosophila. Cell 1994; 7, 79(1):35–47.*
27. *Ward, S. Chemotaxis by the nematode Caenorhabditis elegans: Identification of attractants and analysis of the response by use of mutants. Proc. Natl. Acad. Sci. USA 1973; 70:817-21.*
28. *Ward S., Thomson J. N., White J. G., Brenner S. Electron microscopical reconstruction of the anterior sensory anatomy of the nematode C. elegans. Journal of Comparative Neurology 1975; 160:313-337.*
29. *Wen, J., Kumar, N., Morrison, G., Rambaldini, G., Runciman, S., Rousseau, J. and van der Kooy, D. Mutations that prevent associative learning in C. elegans. Behav. Neurosci. 1997; 111:354-368.*
30. *White J. G., Southgate E., Thomson J. N., Brenner S. The structure of the nervous system of the nematode C. elegans. Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences 1986; 314:1-340.*
31. *Stiernagle T., Maintenance of C. elegans. C. elegans: A practical approach, Oxford University Press, Oxford, 1999, pp 51-67.*