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Acetylcholine Deficiency in *Caenorhabditis elegans* Induced by Hyperthermia Can Be Compensated by ACh-esterase Inhibition or Activation of GAR-3 mAChRs

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Abstract

The nervous system is a target of hyperthermic failure of animal behavior. *Caenorhabditis elegans* can be used as an excellent model organism to investigate mechanisms underlying thermotolerance of nervous system. Inhibition of ACh-esterase by neostigmine produces rise in thermotolerance of *C. elegans* swimming induced by mechanical stimulus at constant temperature 36 °C. Protection of *C. elegans* behavior against heat stress by neostigmine indicates that hyperthermia induces ACh deficiency in the *C. elegans* nervous system which is one of the causes of hyperthermic failure of behavior. Activation of mAChRs by pilocarpine or oxotremorine M elevates behavior thermotolerance similarly with neostigmine while inhibition of these receptors by atropine has opposite effect. These results suggest that ACh protects *C. elegans* behavior against hyperthermia by binding with mAChRs. It is known that three G-protein coupled ACh receptors of *C. elegans* (GAR-1, GAR-2 and GAR-3) have sequence homology with five known subtypes of mammalian mAChRs. To identify mAChRs responsible for regulation of behavior response to hyperthermia we investigated effects of loss-of-function mutations in *gar-1*, *gar-2* and *gar-3* genes on the sensitivity of behavior thermotolerance to neostigmine and pilocarpine. Among them only loss-of-function *gar-3* mutation caused insensitivity of *C. elegans* behavior thermotolerance to neostigmine and pilocarpine. Thus it is GAR-3 mAChR that mediates rise in behavior thermotolerance produced by ACh-esterase inhibitor neostigmine or agonists of mammalian mAChRs.

Keywords: *Caenorhabditis elegans*, behavior thermotolerance, acetylcholine, acetylcholine esterase, GAR-3 muscarinic cholinoreceptor

1. Introduction

Temperature is one of the most important variables that determines distribution and abundance of species (Cossins & Bowler, 1987; David, Allemand, Van Herrewege, & Cohet, 1983; Hoffmann, Sørensen, & Loeschke, 2003). Each invertebrate species tends to have own temperature niche with a distinct optimum and a range of permissible temperatures (David et al., 1983). Nevertheless certain threshold temperatures generally limit reproduction and development in most tropical and temperate species when invertebrates are continuously exposed to constant temperatures (Cossins & Bowler, 1987; David et al., 1983; Hoffmann et al., 2003). At extreme high temperature cells and tissues died. Long before this point, however, invertebrates' organisms experiencing hyperthermia endangered by impaired neural performance that prevents coordinated behavior and hampers vital motor patterns (Robertson, 2004a, 2004b). In humans heat stroke is characterized by central nervous system dysfunction that results in delirium, convulsions and coma (Bouchama & Knochel, 2002). It is known that many invertebrates maintain their behaviors at great elevation of environmental temperature above their temperature optimum (Hoffmann et al., 2003; Robertson, 2004a, 2004b). Therefore it is evident that nervous systems of free-living invertebrates are particularly susceptible to detrimental direct effects of heat stress, as their body temperature is determined to a large extent by environmental temperature.

The possible mechanisms of neural circuits dysfunction caused by extreme high temperature are very complex

because of high sensitivity to elevated temperature of all processes taking place at all levels of nervous system organization.

In the electrophysiological investigations hyperthermic disturbances of neural circuits are revealed in the disturbances of synaptic transmission (Barclay & Robertson, 2000, 2001; Dawson-Scully & Robertson, 1998; Kelty, Noseworthy, Feder, Robertson, & Ramirez, 2002), and prior adaptation to the thermal extremes by thermal preconditioning can improve synaptic thermoprotection (Barclay & Robertson, 2000, 2001; Dawson-Scully & Robertson, 1998; Kelty et al., 2002). At the present time it is evident that the nervous system is a target for hyperthermic failure of invertebrates behavior (Robertson, 2004b; Kalinnikova, Kolsanova, & Gainutdinov, 2012) Therefore hyperthermic failure of different forms of invertebrate's behavior can be used for investigation of mechanisms, which determine both impair of the whole nervous system functions and protection of these functions against hyperthermia.

Since all types of synaptic transmission are sensitive to hyperthermia it is evident that all of them can be potential targets for heat stress effects. However it is possible that role of separate types of synaptic transmission in the whole nervous system dysfunction produced by heat stress can be different. Cholinergic synaptic transmission plays extremely important role in the large majority of functions of human and animals' organisms including thermoregulation (Conti-Tronconi & Raffery, 1982; Ellis et al., 2006; Erskine et al., 2004; Feiro & Gould, 2005; Gomeza et al., 1999; Lanzafame, Christopoulos, & Mitchelson, 2003). Therefore it is possible that cholinergic system plays important role in the response of whole nervous system to hyperthermia including its dysfunctions and compensatory responses.

The aim of this work was to check the hypothesis assuming that cholinergic system is a target for hyperthermic failure of invertebrates' behavior.

However such investigations using organisms of higher invertebrates as a model have many difficulties: (i) complexity of higher invertebrates' organisms and their nervous system which include neural circuits regulating locomotion, respiration, circulation and other functions of multicellular organism; (ii) hematoneural barrier of higher invertebrates is poor permeable for many chemicals which can be used for neuropharmacological analysis of behavior under optimal or stressful conditions. That's why we have proposed that more simple organism of microscopic soil nematode *Caenorhabditis elegans* can be used as a model to investigate mechanisms of the nervous system thermotolerance. *C. elegans* has several advantages in comparison with higher invertebrates: (i) the absence of circulatory and respiratory systems, and consequently neural circuits which regulate these systems in the organisms of higher invertebrates and can be the targets of hyperthermia effect (Armstrong, Shoemaker, Money, & Robertson, 2006); (ii) the absence of hematoneural barrier, which protect neural circuits of higher invertebrates against chemicals from internal environment and thus complicates neuropharmacological analysis of nervous system's thermotolerance in behavioral tests; (iii) the nervous system of the adult hermaphrodite consists of only 302 neurons subdividing into 118 types and for many years is used as an ideal model to investigate genetic, molecular and cellular mechanisms of nervous system's functions (Bargmann, 1993). Therefore molecular and cellular mechanisms of many forms of *C. elegans* behavior are known (Chase & Koelle, 2007; Hart, 2006), and this knowledge can be used for investigation of *C. elegans* behavior under hyperthermic conditions.

Acetylcholine (ACh) is the major neurotransmitter not only in vertebrates but also in the simple organism of soil nematode *Caenorhabditis elegans* and more than one third of neurons in *C. elegans* nervous system release ACh. In *C. elegans* molecular mechanisms of ACh effects on postsynaptic neurons and muscles are similar with such in mammals and are realized by ACh binding with either nicotinic or muscarinic receptors (Changeux & Edelstein, 1998; Fleming, et al., 1997; Kim, Shin, Park, & Cho, 2008; Lee et al., 2000; Liu, LeBoeuf, & Garcia, 2007; Park, Kim, Shin, Choi, & Cho, 2003; Satelle, 2009; Steger & Avery, 2004). Nicotinic receptors (nAChRs) are ligand-gated ion channels and responsible for the initial fast depolarization in the postsynaptic neurons and muscles (Changeux & Edelstein, 1998; Culetto et al., 2004; Satelle, 2009; Unwin, 2005). Muscarinic receptors (mAChRs) are coupled with variety of G-proteins and thereby extend neurotransmission into multiple intracellular signaling processes (Caulfield & Birdsall, 1998; Langmead, Watson, & Reavill, 2008). In order to reveal the possible role of cholinergic system in the *C. elegans* thermotolerance we have examined effects of ACh-esterase inhibitor neostigmine, agonists of nAChRs and mAChRs and antagonist of mAChRs atropine on thermostability of *C. elegans* swimming induced by mechanical stimulus. The possible role of mAChRs in behavior thermotolerance was examined by comparison of behavior thermotolerance sensitivity to neostigmine and agonist of mAChRs pilocarpine of wild-type worms and worms with loss-of-function mutations of three mAChRs genes (*gar-1*, *gar-2* and *gar-3*). These studies showed that hyperthermia causes in *C. elegans* organism ACh deficiency which is revealed in the lowering of behavior thermotolerance and can be compensated both by

ACh-esterase inhibition and action of mAChRs agonists. In addition it was shown that compensation of ACh deficiency by ACh-esterase inhibition or by mAChRs agonists is a result of activation of GAR-3 mAChRs.

2. Methods

2.1 Worms' Strains And Growth

Caenorhabditis elegans were grown at 22 °C in Petri dishes with standard Nematode Growth Medium (NGM) (3 g/l NaCl, 17 g/l Bactoagar, 2.5 g/l Bactopeptone, 1 ml/l, 5 mg/ml cholesterol, 1 ml/l 1 M $CaCl_2$, 1 ml/l 1 M $MgSO_4$, 25 ml/l potassium phosphate buffer [pH6.0]) seeded with E.coli OP50 (Brenner, 1974). The following strains were used in this study: N2 Bristol, *gar-1 (ok755)*, *gar-2 (ok520)* and *gar-3 (vu78)*. All strains were received from Caenorhabditis Genetics Center. *gar-1 (ok755)* and *gar-2 (ok520)* were generated by *C. elegans* gene Knockout Consortium. N2 Bristol is a wild type strain used in numerous genetic and molecular physiology investigations of *C. elegans*. Mutant strains *gar-1 (ok755)*, *gar-2 (ok520)* and *gar-3 (vu78)* are strains with loss-of-function mutations of one of three genes coding *C. elegans* mAChRs GAR-1, GAR-2 and GAR-3 respectively.

2.2 Behavior Thermotolerance Assays

Experiments on measuring the behavior stability to heat stress action were performed in NG buffer (0.3 % NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM, pH 6.0, potassium phosphate/liter) with young adult hermaphrodites from 3-days old culture of worms. For each experiment worms were washed from agar surface into Petri dish 40 mm in diameter and then transferred with pipette into glass centrifuge tube. In this tube worms were rinsed from growth medium, bacteria and metabolites. For this purpose 10 ml of NG buffer was added into tube. After worms' settling on tube's bottom the supernatant was removed. This procedure was repeated three times. The total rinse time was about 30 minutes. After such procedure worms were transferred into clean Petri dish 40 mm in diameter with NG buffer and then transferred with pipette 10 μl into glass tubes with 1 ml of NG buffer (one worm in each tube). To measure thermotolerance of behavior tubes with worms were placed into water bath with temperature 36 °C immediately or after preadaptation to elevated temperature by short-term (120 minutes) exposure to constant temperature 30 °C tolerated by *C. elegans*. The indexes used to characterize behavioral stability under exposure to a constant high temperature 36 °C were as follows: (i) the average exposure time leading to the appearance of uncoordinated behavior (swimming induced by a mechanical stimulus (shaking of the tube with the nematode)), and (ii) the average exposure time leading to loss of worms' ability to swimming induced by a mechanical stimulus. The signs of uncoordinated behavior were: (i) partial incoordination of body muscle contraction necessary for sinusoidal body movements; (ii) inability to sustained forward swimming during 10 seconds after mechanical stimulus.

2.3 Neuropharmacological Analysis of Behavior Thermotolerance

For neuropharmacological analysis of behavior thermotolerance were used inhibitor of ACh-esterase neostigmine bromide, agonists of nAChRs levamisole hydrochloride and nicotine hemisulfate, non-selective agonist of ACh receptors carbamylchloride chloride (carbachol), agonists of mAChRs pilocarpine nitrate and oxotremorine methiodide (oxotremorine M) and mAChRs antagonist atropine methyl bromide. In all experiments were used freshly prepared solutions of reagents. All substances were dissolved in distilled water and added to tubes with worms just before the exposure to temperature 36 °C or 23 °C in control experiments. Reagents were obtained from Sigma.

It is necessary to note that concentrations of drugs and toxicants used in our neuropharmacological analysis of *C. elegans* thermotolerance in most cases were very high (10^{-3} M or more). Such high concentrations are explained by well known specificity of *C. elegans* as a model organism. *C. elegans* organism has very low sensitivity to most chemicals from environment because its cuticle is extremely impermeable to most organic and inorganic chemicals. Therefore concentrations of drugs and toxicants effective for changes of *C. elegans* behavior are very high (Anderson, Cole, & Williams, 2004; Boyd, Cole, Anderson, & Williams, 2003; Carnell, Illi, Hong, & McIntire, 2005; Davies et al., 2003; Davies, Bettinger, Thiele, Judy, & McIntire, 2004; Johnson & Nelson, 1991; Nurrish, Ségalat, & Kaplan, 1999; Sawin, Ranganathan, & Horvitz, 2000; Schafer & Kenyon, 1995; Singer, Bellingham, & Berger, 1996; Tissenbaum et al., 2000).

All experiments presented in this paper were performed in September, October and November. Each datum on the graphics is the mean thermotolerance for 20 worms incubated individually. In each case were performed 4 or 5 independent experiments with similar results, but in the article are shown results of only one of these experiments.

3. Results

Locomotion is the most important ACh-mediated behavior of *C. elegans*, involving by far the greatest number of cholinergic neurons. The involvement of ACh in *C. elegans* locomotion includes not only neuromuscular transmission, but also neuron-neuron transmission (Culetto et al., 2004; Esmaeili, Ross, Neades, Miller, & Ahringer, 2002; Satelle, 2009). Therefore we propose that cholinergic signaling may be one of the major targets of hyperthermia effect on *C. elegans* swimming, induced by mechanical stimulus. To check this hypothesis we tested the influence of drugs affecting cholinergic signaling on *C. elegans* ability to maintain swimming induced by mechanical stimulus at constant high temperature 36 °C.

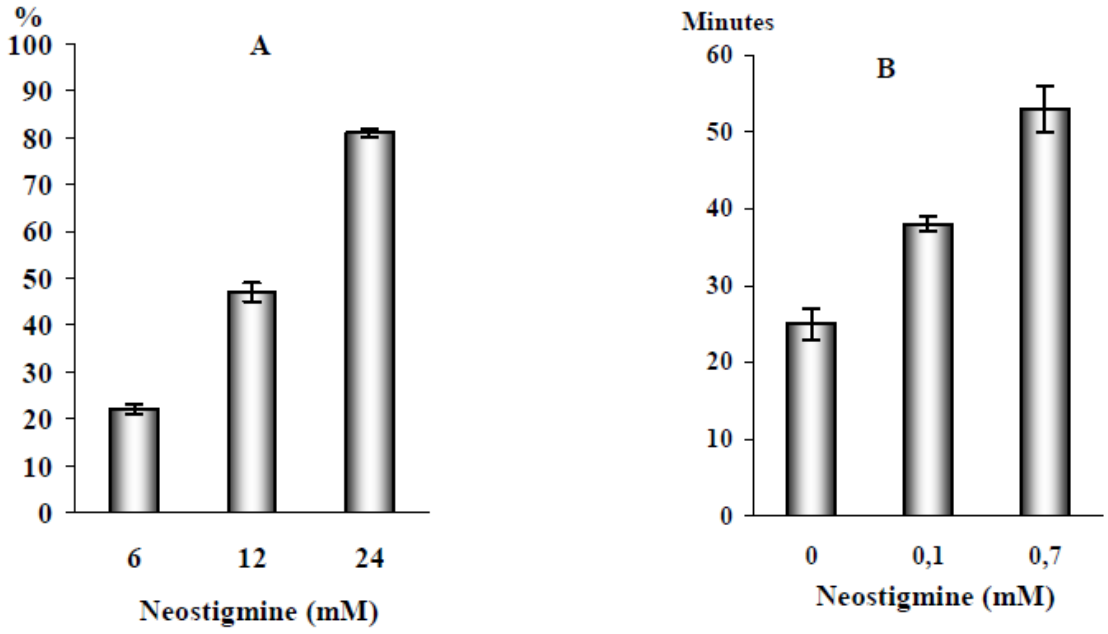

Figure 1. Toxic and "therapeutic" effects of neostigmine on *C. elegans* behavior

A – The ordinate shows the percentage of worms with uncoordinated behavior (swimming, induced by mechanical stimulus) after 90-minute exposition to neostigmine at 22 °C. The abscissa shows neostigmine concentration (mM).

B – The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows neostigmine concentration (mM).

The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Twenty nematodes of wild type strain N2 were used in each variant of experiment.

Neostigmine is a reversible inhibitor of ACh-esterase, and its effects on mammals' organisms are mediated by the rise of ACh (Harvey, Champe, Finkel, Cubeddu, & Clark, 2009). It is known that inhibition of ACh-esterase by strong inhibitors such as aldicarb, used as pesticide, is very toxic not only for insects and mammals, but also for *C.elegans* (Govorunova et al., 2010; Nurrish et al., 1999). However neostigmine and other reversible inhibitors of ACh-esterase are used for therapy of many disorders linked to a deficiency of ACh (Bartus, Dean, Beer, & Lippa, 1982; Harvey et al., 2009; Perry et al., 1978; Tabet, 2006). In our experiments aldicarb-like toxic effects of neostigmine, such as worms' paralysis were revealed only at very high concentrations of this drug (6 mM and above) at normal temperature (22 °C) (Figure 1A). However at lower concentrations (0.1-0.7 mM) neostigmine caused rise in behavior thermotolerance in the dose-dependent manner (Figure 1B). This therapeutic effect of neostigmine was revealed in the rise of mean time course of the worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at constant high temperature 36 °C. Protection of *C. elegans* behavior against heat stress by neostigmine was shown both for basal thermotolerance and for

thermotolerance induced by short-term adaptation of worms to high temperature, such as 2-hour exposure to temperature 30 °C (Figure 2). The dependence of behavior thermotolerance from ACh rise caused by ACh-esterase inhibition shows, that one of hyperthermia effects responsible for behavior failure is ACh deficiency in the *C. elegans* organism.

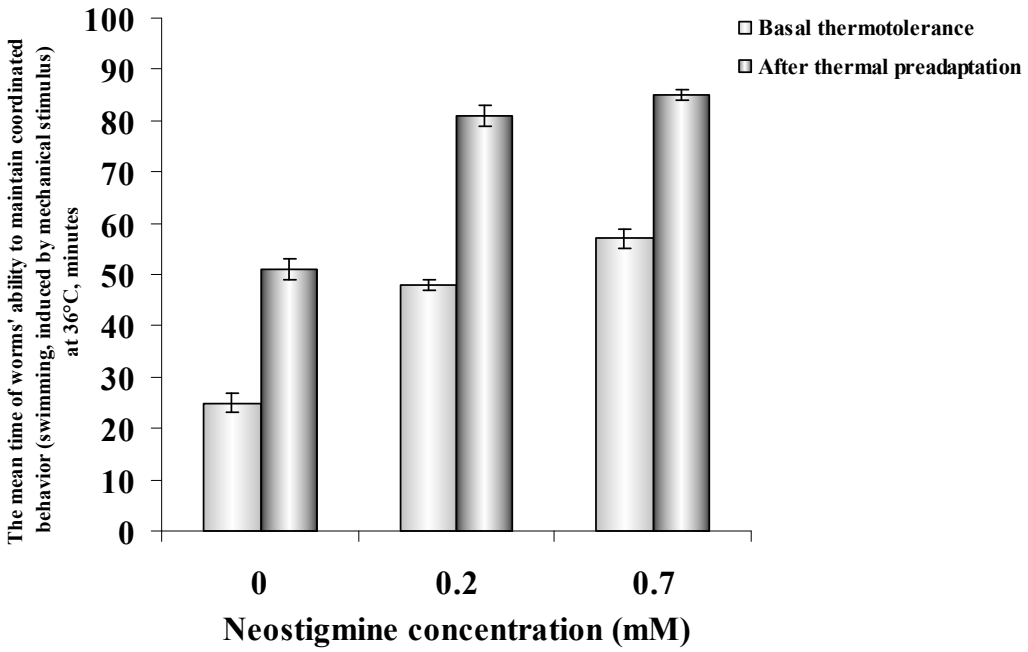

Figure 2. The dependence of *C. elegans* thermotolerance sensitivity to neostigmine from prior adaptation to elevated temperature

The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows neostigmine concentration (mM). The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Prior adaptation to high temperature consisted in 2-hours incubation of individual worms in 1 ml of liquid medium at 30 °C. Twenty nematodes of wild type strain N2 were used in each variant of experiment.

Fast excitatory cholinergic signaling is mediated by activation of nicotinic acetylcholine receptors (nAChRs) in neurons and muscles of *C. elegans* (Culetto et al., 2004; Esmaeili et al., 2002; Satelle, 2009). Therefore the rise in *C. elegans* behavior thermotolerance caused by ACh-esterase inhibition (Figure 1B, 2) can be a consequence of nAChRs overactivation. The *C. elegans* cholinergic signaling contains pharmacologically distinct subtypes of nAChRs: levamisole-sensitive nAChRs and levamisole-insensitive nAChRs (Satelle, 2009). That is why we tested effects both of nicotine and levamisole on time course of failure of worms' swimming induced by mechanical stimulus at constant high temperature 36 °C. As shown in Figure 3, both nicotine and levamisole caused dose-dependent decrease of swimming thermotolerance revealed in the lowering of mean time of loss of worms' ability to swim. Nicotine and levamisole reduced *C. elegans* thermotolerance in concentrations 0.03% and 16 μM consequently. These concentrations of nicotine and levamisole did not cause the loss of worms' ability to swimming induced by mechanical stimulus at temperature 22 °C (data not shown). These data indicate that overactivation of nAChRs can not be a mechanism of thermotolerance elevation by neostigmine. Moreover, it sensitizes *C. elegans* behavior to hyperthermia.

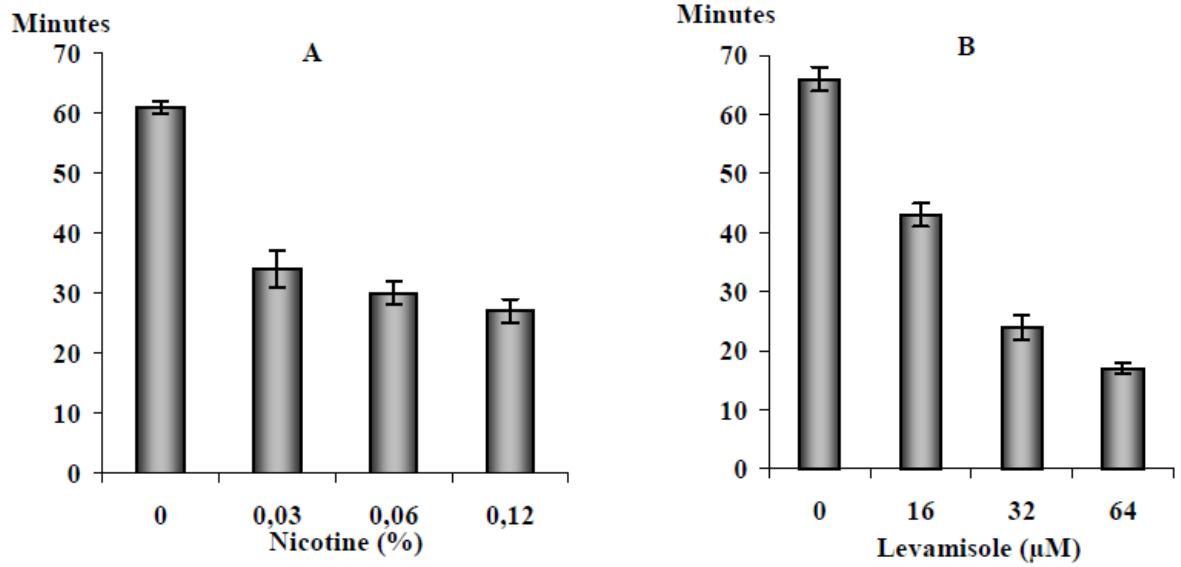

Figure 3. The influence of nicotine and levamisole on thermotolerance of *C. elegans* behavior

The ordinate shows the mean time of worms' ability to maintain swimming, induced by mechanical stimuli at 36 °C. In these experiments was registered complete loss of worms' ability to swimming. The abscissa shows: A – concentration of nicotine (%); B – concentration of levamisole (μM). Twenty nematodes of wild type strain N2 were used in each variant of experiment.

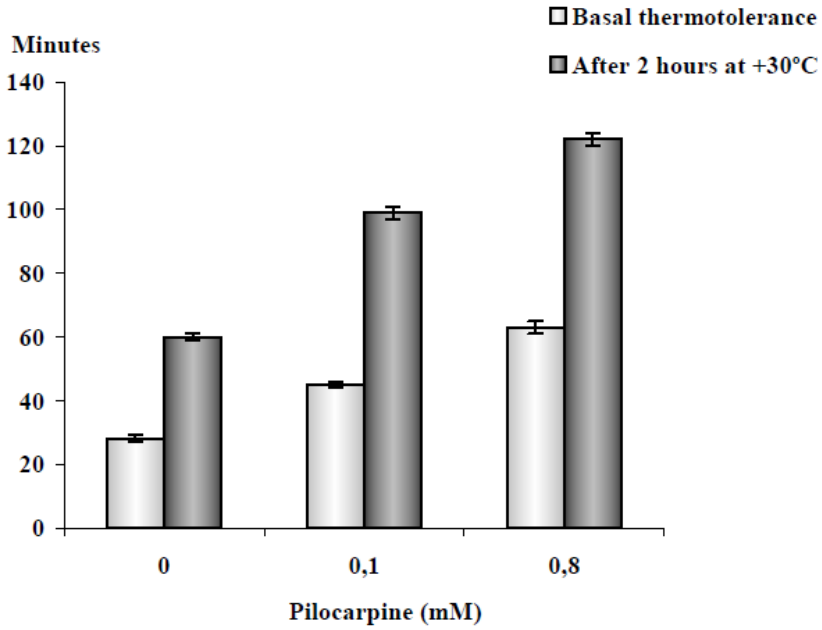

Figure 4. The effect of pilocarpine on *C. elegans* behavior thermotolerance

The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows pilocarpine concentration (mM).

The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Prior adaptation to high temperature consisted in 2-hours incubation of individual worms in 1 ml of liquid medium at 30 °C. Twenty nematodes of wild type strain N2 were used in each variant of experiment.

Actions of acetylcholine on neurons and muscles of *C. elegans* are the result of activation not only nAChRs, but also metabotropic muscarinic acetylcholine receptors (mAChRs) (Kim et al., 2008; Lee et al., 2000; Liu et al., 2007; Park et al., 2003; Steger & Avery, 2004). In order to determine the possible role of mAChRs in the behavior thermotolerance we tested the influence of agonists of these receptors on the ability of *C. elegans* to maintain swimming, induced by mechanical stimulus at extreme high temperature 36 °C. Pilocarpine and oxotremorine M are agonists of mammalian mAChRs. As shown in Figures 4 and 5, addition of pilocarpine or oxotremorine M to NG buffer elevated behavior thermotolerance revealed in the rise of mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at extreme high temperature 36 °C. Carbachol, non-selective agonist of mammalian ACh receptors, acts on *C. elegans* behavior thermotolerance similarly with pilocarpine and oxotremorine M (Figure 5). Therapeutic effect of carbachol on *C. elegans* behavior in hyperthermic conditions was completely blocked by addition of mAChRs antagonist atropine (Figures 5, 6). Therefore it is evident that carbachol effect on *C. elegans* behavior thermotolerance is mediated by activation of mAChRs. Data in Figures 5 and 6 show that atropine reduced swimming thermotolerance not only in the presence, but also in the absence of carbachol in the medium. This effect of atropine can be a consequence of blocking of mAChRs activation by endogenous ACh. The data in Figure 6 show that atropine strongly attenuated protection of behavior thermotolerance caused by neostigmine. These data support that activation of mAChRs is the general mechanism of the thermotolerance rise caused by inhibition of ACh-esterase.

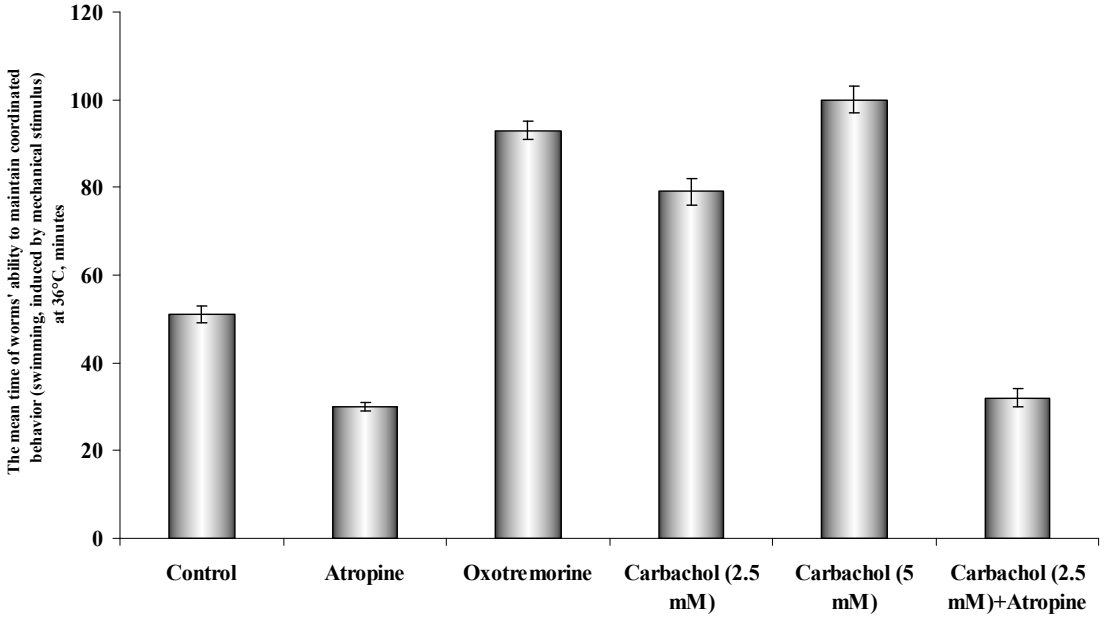

Figure 5. The effects of atropine and mAChRs agonists on *C. elegans* behavior thermotolerance

The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows conditions of experiments. Drugs concentrations were as follows: atropine – 1.5 mM, oxotremorine – 5.0 mM, carbachol – 2.5 or 5 mM.

The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Twenty nematodes of wild type strain N2 were used in each variant of experiment.

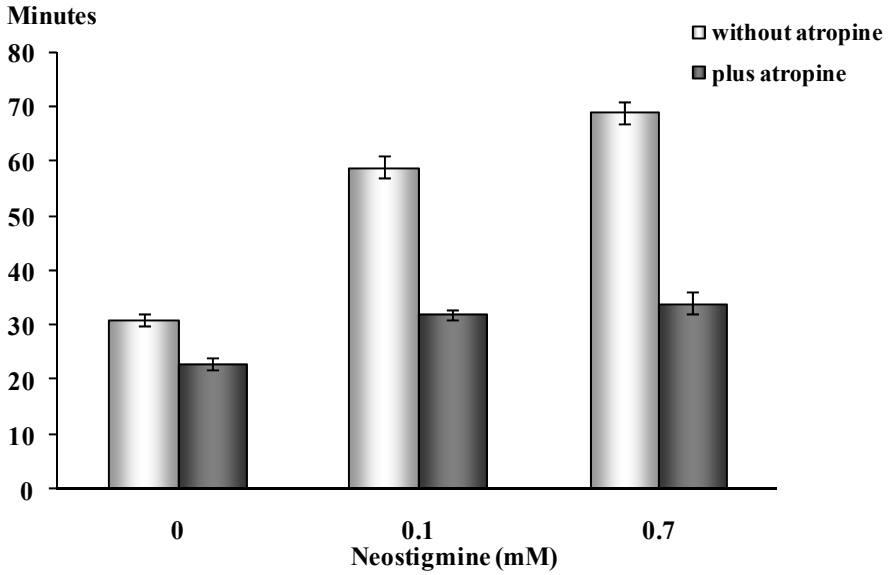

Figure 6. The effects of neostigmine and atropine on *C. elegans* behavior thermotolerance

The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows concentrations of neostigmine (mM). Atropine concentration was 1.5 mM.

The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Twenty nematodes of wild type strain N2 were used in each variant of experiment.

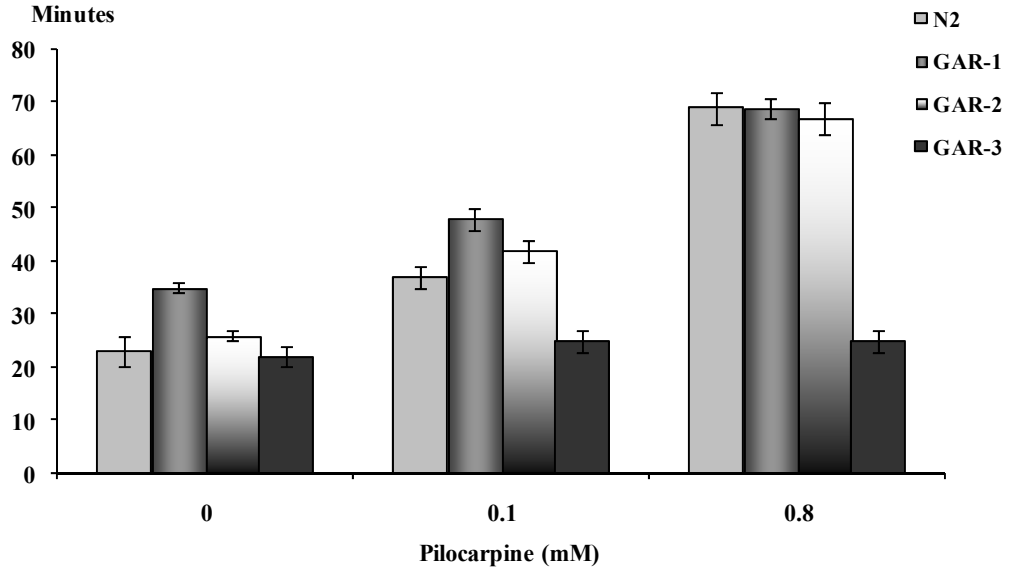

Figure 7. Thermotolerance sensitivity to pilocarpine of C. elegans wild type strain N2 and three mutant strains: *gar-1(ok755)*, *gar-2(ok520)* and *gar-3(vu78)*

The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows pilocarpine concentration (mM).

The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Twenty nematodes were used in each variant of experiment.

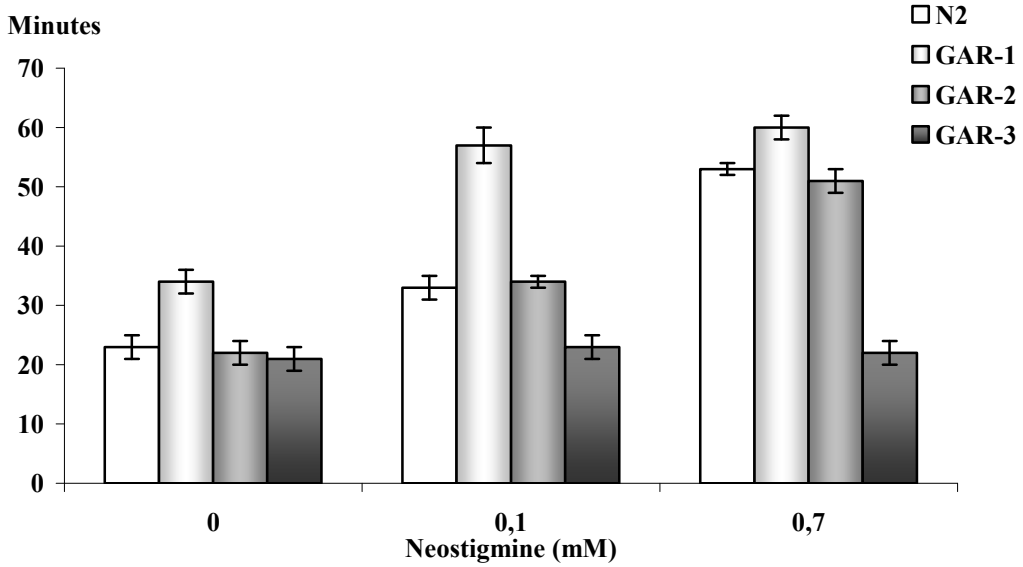

Figure 8. Thermotolerance sensitivity to neostigmine of *C. elegans* wild type strain N2 and three mutant strains: *gar-1(ok755)*, *gar-2(ok520)* and *gar-3(vu78)*

The ordinate shows the mean time of worms' ability to maintain coordinated behavior (swimming, induced by mechanical stimulus) at 36 °C. The abscissa shows neostigmine concentration (mM).

The signs of uncoordinated behavior were as follows: (i) inability to sustained forward swimming during 10 seconds after mechanical stimulus; (ii) partial incoordination of body muscle contraction necessary for sinusoidal body movements. Twenty nematodes were used in each variant of experiment.

The *C. elegans* genome encodes three mAChRs, GAR-1, GAR-2 and GAR-3 (Kim et al., 2008; Lee et al., 2000; Liu et al., 2007; Park et al., 2003; Steger & Avery, 2004). All of them show high similarity in amino acids sequences to the known mammalian mAChRs M_1, M_2, M_3, M_4 and M_5, which are coupled to different G-proteins (Caulfield & Birdsall, 1998; Langmead et al., 2008; Lanzafame et al., 2003). To identify mAChRs responsible for the rise in behavior thermotolerance we analyzed available deletions mutants of all of three genes of these receptors by estimation of thermotolerance sensitivity to agonist of mAChRs pilocarpine and inhibitor of ACh-esterase neostigmine. The behavior thermotolerance in the absence of pilocarpine or neostigmine was not significantly changed in *gar-2* or *gar-3* loss-of-function mutants, while *gar-1* loss-of-function mutant was more resistant to constant high temperature 36 °C in comparison with that of wild type strain N2 (Figures 7, 8). In contrast, sensitivity of the behavior thermotolerance to pilocarpine was not significantly changed in *gar-1* or *gar-2* mutants, but loss-of-function mutation of *gar-3* gene almost completely ceased protection of worms' behavior against heat stress caused by pilocarpine (Figure 7). Similar data were obtained in experiments, in which we compared the sensitivity of behavior thermotolerance to neostigmine of N2 strain and *gar-1*, *gar-2* and *gar-3* mutants. While neostigmine induced rise in behavior thermotolerance of *gar-1* and *gar-2* mutants with dose-dependent response similar to this of N2 worms, the sensitivity of behavior thermotolerance in *gar-3* mutants to neostigmine was very slight (Figure 8). These data indicate that *gar-3* function is required for effects of both agonist of mAChRs pilocarpine and elevated endogenous ACh concentration on behavior thermotolerance of *C. elegans*.

4. Discussion

We have showed that reversible inhibitor of ACh-esterase neostigmine (Harvey et al., 2009) protected *C. elegans* behavior against hyperthermia (Figures 1, 2). Inhibition of the ACh-esterase activity prevents ACh hydrolysis and, as a result, elevates ACh concentration and prolongs its action on the postsynaptic receptors. Effects of ACh-esterase inhibitors on the functions of the whole human or animal organism can be either toxic (Govorunova et al., 2010; Nurrish et al., 1999) or therapeutic (Bartus et al., 1982; Harvey et al., 2009; Perry et al., 1978; Tabet, 2006). In the first case ACh inhibitors disturb cholinergic transmission by enormous rise of ACh concentration resulting in overactivation of postsynaptic AChRs. Such enormous rise of ACh concentrations leads to the paralysis or even death of organism (Govorunova et al., 2010; Nurrish et al., 1999). On the other

hand, inhibition of ACh-esterase can be therapeutic for the organism if the complementary rise of ACh concentration is required for the compensation of ACh deficiency which has been established a core pathophysiological feature in Alzheimer's disease, Parkinson's disease, vascular dementia and multiple sclerosis's dementia (Bartus et al., 1982; Harvey et al., 2009; Perry et al., 1978; Tabet, 2006). Therefore inhibitors of ACh-esterase are used in agriculture as pesticides and in medicine as drugs for neurologic disorders. In studies of cholinergic signaling in *C. elegans* for inhibition of ACh-esterase usually is used aldicarb. Aldicarb is known as pesticide caused enormous rise of ACh in the neuron-muscle ACh transmission revealed in worm's paralysis (Govorunova et al., 2010; Nurrish et al., 1999). In contrast, reversible inhibitor of ACh-esterase neostigmine is usually used in medicine and neurophysiological studies of mammals (Harvey et al., 2009; Naguib & Yaksh, 1997). In our experiments with *C. elegans* aldicarb-like toxic effect of neostigmine was shown for concentrations 60-fold higher than those effective for its therapeutic effect revealed in the rise of behavior thermotolerance (Figure 1). Since therapeutic effects of ACh-esterase inhibitors are shown only in the ACh deficiency states of cholinergic system (Bartus et al., 1982; Harvey et al., 2009), protection of *C. elegans* behavior against heat stress by neostigmine (Figures 1, 2) indicates that one of consequences of hyperthermia action is the deficit of ACh in *C. elegans* organism. Three possible reasons of ACh deficit caused by hyperthermia are: (i) inhibition of ACh secretion by cholinergic neurons; (ii) increase of ACh hydrolysis by ACh-esterase caused by extreme high temperature; (iii) requirement of supplementary ACh for compensation of other disturbances caused by hyperthermia in nervous system. Independently of the reason of ACh deficiency in *C. elegans* organism in hyperthermic conditions it is evident that this deficiency is one of reasons of behavior disturbances caused by heat stress, since these disturbances are attenuated by neostigmine (Figures 1, 2).

It is known that resistance of poikilotherms' behavior to thermal extremes can be increased by prior adaptation to elevated temperature. Long-term exposure to constant elevated temperature within the normal viable temperature range of an organism is usually termed "acclimation" (Cossins & Bowler, 1987). Since ACh deficiency is one of reasons of behavior disturbances caused by hyperthermia the possible mechanism of that prior adaptation to high environmental temperature can be prevention or lowering of this deficiency. In this case the sensitivity of *C. elegans* behavior thermotolerance to ACh-esterase inhibitor must be diminished or completely lost after prior adaptation to constant high temperature tolerated by worm's organism. However, effects of prior adaptation to elevated temperature 30 °C and neostigmine on behavior thermotolerance are additive or slightly synergic (Figure 3). Therefore it is evident that prior adaptation to high environmental temperature, used in this work, did not prevent ACh deficiency induced by hyperthermia although it is possible that it extends time-course of its appearance.

ACh effects on excitable cells are mediated by its binding with two types of receptors, namely nAChRs and mAChRs (Culetto et al., 2004; Esmaeili et al., 2002; Kim et al., 2008; Lee et al., 2000; Liu et al., 2007; Park et al., 2003; Steger & Avery, 2004; Satelle, 2009). Therefore, two possible mechanisms of behavior protection against hyperthermia by ACh rise due to inhibition of ACh-esterase are overactivation of nAChRs or overactivation of mAChRs. Cholinergic system of *C. elegans* consists of two subtypes of nAChRs, namely levamisole-sensitive and levamisole-insensitive (Satelle, 2009). Both levamisole and nicotine did not protect *C. elegans* behavior against hyperthermia but evoked the opposite effect - sensitization of *C. elegans* behavior to high extreme temperature (Figure 3). Therefore overactivation of L-subtype or N-subtype nicotinic receptors can not be a mechanism of the protection of *C. elegans* behavior against heat stress via rise of ACh content in its organism. Moreover it is possible that overactivation of nAChRs not only by nicotine or levamisole but also by enormous rise of ACh can diminish thermotolerance of *C. elegans* behavior.

In contrast to effects of agonists of nAChRs, agonists of mammalian mAChRs pilocarpine and oxotremorine M mimic protective effect of neostigmine on *C. elegans* behavior in hyperthermia conditions while antagonist of mammalian mAChRs atropine had opposite effect and greatly attenuated protective effect of neostigmine (Figures 4-6). These results suggest that ACh protects *C. elegans* behavior against hyperthermia by activation of mAChRs.

Muscarinic acetylcholine receptors are known to regulate numerous fundamental physiological processes including muscarinic actions of acetylcholine on peripheral effector tissues and a multitude of central sensory, vegetative and motor functions (Ellis et al., 2006; Erskine et al., 2004; Feiro & Gould, 2005; Lanzafame et al., 2003). Many studies have shown that central muscarinic receptors play an important role in the regulation of body temperature in mammals (Gomeza et al., 1999) and therefore are necessary for survival of these animals and humans at hot environment. However the possible role of mAChRs in the thermotolerance of poikilotherms is still unknown.

The *C. elegans* genome encodes three mAChRs, namely GAR-1, GAR-2 and GAR-3 (Kim et al., 2008; Lee et

al., 2000; Liu et al., 2007; Park, Kim et al., 2003; Steger & Avery, 2004). All of them show high similarity in amino acid sequences to the known mammalian mAChRs M_1, M_2, M_3, M_4 and M_5, which are coupled to different G-proteins (Caulfield & Birdsall, 1998; Langmead et al., 2008; Lanzafame et al., 2003).

Results of pharmacological analysis of *gar-1*, *gar-2* and *gar-3* ectopically expressed in cell cultures (Kim et al., 2008; Lee et al., 2000; Park et al., 2003) and data presented in this paper show that activation of GAR-3 mAChRs is a general mechanism of the increase of *C. elegans* behavior thermotolerance by agonists of mammalian mAChRs or by rise of ACh content as a result of ACh-esterase inhibition: (i) loss-of-function *gar-3* mutants are insensitive to pilocarpine and neostigmine effects on behavior thermotolerance while the sensitivity of loss-of-function *gar-1* and *gar-2* mutants to this substances at hyperthermia conditions is comparable with such of wild type worms (Figures 7, 8); (ii) the sensitivity of cultured cells to agonists of mammalian mAChRs can be induced by ectopic expression of *gar-3* but not *gar-1* or *gar-2* (Kim et al., 2008; Lee et al., 2000; Park et al., 2003). Therefore the sensitivity of behavior thermotolerance to such agonists of mammalian mAChRs, as pilocarpine, oxotremorine M and carbachol (Figures 4, 5) can be a consequence of activation of GAR-3 mAChRs but not of GAR-1 or GAR-2 mAChRs; (iii) only *gar-3* expression in cultured cells produced significant sensitivity to non-selective antagonist of mammalian mAChRs atropine while cells with *gar-1* expression had only slight sensitivity to atropine and cells with *gar-2* expression were completely insensitive to atropine (Lee et al., 2000). Therefore the most possible explanation of decrease of *C. elegans* thermotolerance by atropine (Figures 5, 6) is the block of GAR-3 muscarinic receptors activation by ACh.

gar-3 is expressed in many neurons and peripheral tissues of *C. elegans* including body wall muscles (Liu et al., 2007; Steger & Avery, 2004). It is known that GAR-3 muscarinic receptor regulates feeding and male mating behaviors of *C. elegans* (Liu et al., 2007; Steger & Avery, 2004). However possible role of GAR-3 in *C. elegans* locomotion is not revealed. Moreover phenotypes of *gar-3* loss-of-function mutants are almost wild-type with exception of faster pharyngeal pumping (Steger & Avery, 2004), suggesting that GAR-3 does not regulate *C. elegans* locomotion under optimal temperature conditions. However, as it is shown in this article, GAR-3 can play important role in the maintenance of *C. elegans* locomotion at extreme high temperature.

The major question has arisen from our data is why loss-of-function *gar-3* mutants in conditions without overactivation of mAChRs either by exogenous agonists or by the rise of endogenous ACh content due to ACh-esterase inhibition have behavior thermotolerance similar with such of wild type worms (Figure 7, 8)? The study of mAChRs knockout animals is an important tool to understanding the role of these receptors in the functions of rodents' and *C. elegans* nervous system. However the physiological relevance of muscarinic signaling is sometimes difficult to pinpoint in these studies. Knockout mice lacking any one of the five mammalian muscarinic receptors subtypes are viable and generally healthy (Gomeza et al., 1999; Hamilton et al., 1997; Zhang et al., 2002). For example M_2 muscarinic receptor knockout mice have normal movement and temperature control, while pharmacological analysis revealed the major role of M_2 mAChR in the thermoregulation and locomotion (Gomeza et al., 1999). The similar data were obtained in experiments with loss-of-function mAChRs mutants of *C. elegans*, since *C. elegans* lacking GAR-2 or GAR-3 mAChRs have generally normal behavior, and differences of all forms of behavior between worms lacking GAR-1 mAChRs and wild type worms are not detectable at optimal temperature (Liu et al., 2007; Steger & Avery, 2004).

It is known that the effect of knockout mutation on animal organism includes both defects of organism functions caused by direct effects of mutations and opposite compensatory effects which allow to remain behavior or other functions of organism in the absence of gene functions necessary for wild type animal (Valet, Tavernier, Castan-Laurell, Sébastien, & Langin, 2002).

Therefore almost normal or fully normal behavior of mutant mice and *C. elegans* lacking one of mAChRs subtypes must be the result of compensatory response of organism on mutation effect. On the other hand the major role of mAChRs in many behaviors in mammals and *C. elegans* is identified by pharmacological analysis of these behaviors using agonists and antagonists of these receptors (Gomeza et al., 1999; Hamilton et al., 1997; Liu et al., 2007; Steger & Avery, 2004; Zhang et al., 2002). Direct effect of single mutation lacking one of mAChRs subtypes was revealed in mice and *C. elegans* in the insensitivity of behavior to agonists and antagonists' action, and this insensitivity allows identifying the possible key role of mAChRs subtypes in the multiple forms of behavior. For example, in mice disruption of M_2 mAChR gene caused great insensitivity of thermoregulation and M-current activity in sympathetic neurons to agonists of mAChRs (Gomeza et al., 1999), and in *C. elegans* loss-of-function *gar-3* mutants are insensitive to effects of mAChRs agonists on feeding and mating behaviors (Liu et al., 2007; Steger & Avery, 2004). Therefore insensitivity of behavior thermotolerance to agonists of mAChRs caused by loss-of-function *gar-3* mutation reveals the key role of GAR-3 mAChR in the regulation of *C. elegans* thermotolerance while the absence of this mutation effect on thermotolerance in the

medium without agonists of mAChRs or ACh-esterase inhibitor neostigmine indicates that lacking of *gar-3* gene can be compensated by *C. elegans* organism.

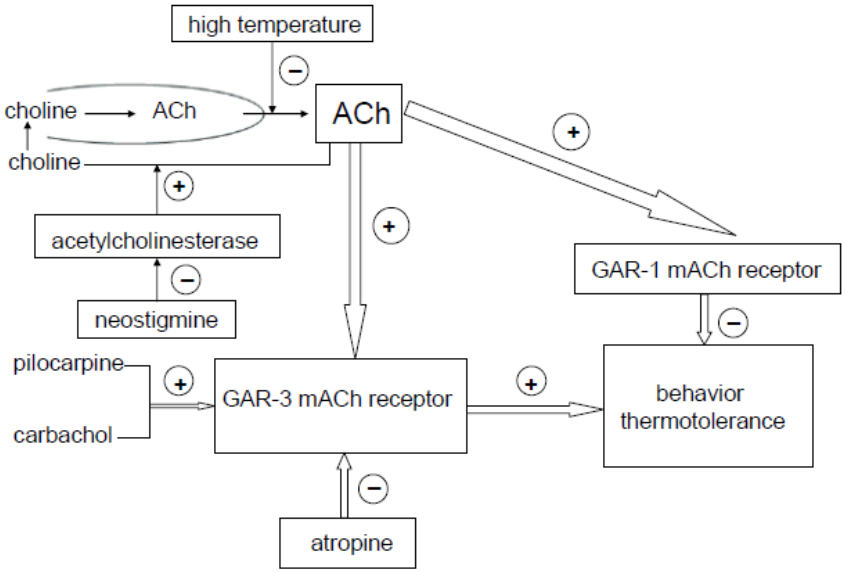

Figure 9. The regulation of *C. elegans* thermotolerance by activation of mAChRs

It is interesting that binding of ACh by GAR-1 mAChRs had opposite to activation of GAR-3 mAChRs effect on behavior thermotolerance since loss-of-function *gar-1* mutation caused rise in *C. elegans* thermotolerance (Figure 7, 8 and scheme in Figure 9). Opposite effects of ACh binding by two subtypes of mAChRs were shown for functions of mammalian and *C. elegans* nervous systems not only at hyperthermia but also at optimal temperature conditions. These effects are explained by differences of expression of genes of mAChRs subtypes in different parts of nervous system and by differences of G-proteins coupled with certain subtypes of mAChRs. For example, in mammals M_1, M_3 and M_5 subtypes of mAChRs are selectively linked to $G_{q/11}$ proteins and activate phospholipase C whereas M_2 and M_4 subtypes are preferentially coupled to $G_{j/0}$ proteins and inhibit adenylate cyclase (Caulfield & Birdsall, 1998; Langmead et al., 2008). Therefore ACh binding by M_2 subtypes receptors in many cases inhibits neurotransmitters release from neurons (Zhang, Chen, & Pan, 2007) while activation of M_1 receptors in contrast stimulates neurotransmitters release (Bauer, Woolley, Teschemacher, & Seward, 2007). In *C. elegans* GAR-2 mAChRs similarly with mammalian M_2 mAChRs mediate negative feedback in cholinergic motor neurons by inhibiting ACh release whereas ectopic expression of *gar-3* in these neurons caused acceleration of ACh secretion (Dittman & Kaplan, 2008).

While the rise in behavior thermotolerance caused by binding of ACh with GAR-3 mAChRs can be explained by stimulation of neurotransmitter release, the opposite effect of ACh binding with GAR-1 mAChRs can not be explained since its physiological relevance is unknown and rise in behavior thermotolerance is the first identified phenotypic feature caused by *gar-1* loss-of-function mutation.

Locomotion is the most important ACh-mediated behavior involving by far the greatest number of cholinergic neurons. The involving of ACh in locomotion includes both neuron-neuron transmission and neuromuscular transmission since ACh is excitatory neurotransmitter depolarizing locomotory muscles. Therefore two possible mechanisms of regulation of locomotion thermotolerance by mAChRs signaling are its effects on the thermostability of neuron-neuron transmission or regulation of thermostability of neuromuscular transmission by ACh binding with GAR-3 or GAR-1 mAChRs. While *gar-3* expression was shown not only in many neurons but also in the body wall muscles (Steger & Avery, 2004), investigation of neuromuscular transmission did not reveal the influence of GAR-3 mAChRs signaling on the sensitivity of locomotory muscles to excitation by nAChRs signaling. However these investigations showed that GAR-3 signaling stimulates neurotransmitter release from cholinergic motor neurons (Dittman & Kaplan, 2008). Therefore it is evident that overactivation of GAR-3 mAChRs can stabilize neuromuscular transmission against heat stress only on presynaptic but not on postsynaptic level if hyperthermia inhibits ACh release from motor neurons. On the other hand, it is possible that

neuron-neuron synaptic transmission in the neural network regulating locomotion is more sensitive to hyperthermia than neuromuscular transmission and in this case agonists of mAChRs can elevate thermotolerance of *C. elegans* locomotion by stimulation of neurotransmitters release from interneurons.

It is known that both neuron-neuron and neuron-muscle synaptic transmissions can be a target for reversible disturbances of invertebrates' behavior caused by hyperthermia (Barclay & Robertson, 2001; Janssen, 1992; Kelty et al., 2002; Robertson, 2004b; Tryba & Ramirez, 2003).

The specificity of our experiments consists in measuring of thermotolerance of swimming induced by strong mechanical stimulus in the absence of sensory inputs into nervous system from food and other worms. Two types of behavior disturbances caused by hyperthermia in these conditions were defects of swimming (the lack of coordination of locomotory muscles necessary for sinusoidal movements) and reversible inability of worms to swimming induced by mechanical stimulus. At constant extreme high temperature the first type of above mentioned swimming defects always precedes the loss of ability to swimming. Three parameters determining swimming thermostability in these conditions are: (i) thermotolerance of mechanosensory system function; (ii) thermotolerance of neuronal network formed by the interneurons AVA, AVB and PVC providing input to the A- and B-type motor neurons (responsible for forward and backward movement) and the inhibitory D-type motor neurons involved in the coordinated movement (Leung et al., 2008); (iii) thermotolerance of neuromuscular synaptic transmission.

It is evident that reversible loss of swimming ability can be explained by cessation of neuromuscular synaptic transmission by hyperthermia. However more weak defects of swimming induced by mechanical stimulus without inability to swimming can be explained only by defects of functions of neural networks regulating *C. elegans* swimming or by defects of mechanosensation. Therefore it is evident that prevention of such weak defects of swimming at extreme high temperature by agonists of mAChRs and ACh-esterase inhibitor is a result of protection of neuron-neuron synaptic transmission against disturbances caused by hyperthermia. This conclusion is in accordance with conception assuming that central processes in the nervous system are the most sensitive targets of negative effects of hyperthermia on invertebrates' behavior (Barclay & Robertson, 2001; Dawson-Scully & Robertson, 1998; Kalinnikova et al., 2012; Kelty et al., 2002; Robertson, 2004b).

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