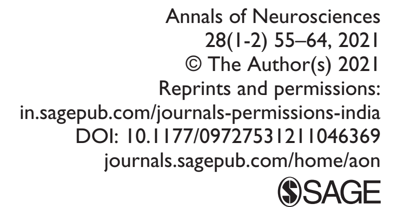
Original Article

DMSO Delays Alzheimer Disease Causing Aβ-induced Paralysis in *C. elegans* Through Modulation of Glutamate/Acetylcholine Neurotransmission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Abstract

Background: Alzheimer's disease (AD), a prevalent neurodegenerative disease with progressive dementia and neurotransmission (NT)-dysfunction-related complications in older adults, is known to be caused by abnormal Amyloid-β (Aβ) peptide and associated amyloid plaques in the brain. Drugs to cure AD are not in sight. Two major excitatory neurotransmitters, glutamate (Glu) and acetylcholine (ACh), and their signaling systems are implicated in AD.

Objective: To determine the effect of various NT-altering compounds including fenobam, quisqualic acid, and dimethyl sulfoxide (DMSO) in the protection against Aβ toxicity. Further, to identify the potential mechanism through which the protection happens.

Methods: The well-known *C. elegans* AD model, CL4176, in which human Aβ expression is turned on upon a temperature shift to 25 °C that leads to paralysis, was screened for protection/delay in paralysis because of **Aβ** toxicity. While screening the compounds, dimethyl sulfoxide (DMSO), a universal solvent used to solubilize compounds, was identified to provide protection. Aldicarb and levamisole assays were performed to identify the contribution of ACh neurotransmission in **Aβ** toxicity protection by DMSO.

Results: One percent and two percent DMSO delayed paralysis by 48% and 90%, respectively. DMSO was dominant over one of the Glu-NT pathway-related compounds, Fenobam-Group I mGluR antagonist. But DMSO provided only 30% to 50% protection against Quisqualic acid, the Glu-agonist. DMSO (2%) delayed ACh-NT, both presynaptic acetylcholine esterase inhibitor (AchEi)-aldicarb and postsynaptic-iAChR-agonst-levamisole induced paralysis, by ~70% in CL4176. DMSO seems to be altering Ca^{2+} ion permeability essential for NT as EthyleneDiamine Tetra-Acetic acid (EDTA) and DMSO provided similar aldicarb resistance either combined or alone in wildtype worms. But postsynaptic Ca^{2+} depletion by EDTA could reverse DMSO-induced levamisole hypersensitivity. Surprisingly, the absence of FOrkhead boX O (FOXO) transcription factor homolog, *daf-16* (loss-of-function mutant), a critical transcription factor in the reduced IIS-mediated longevity in *C. elegans,* abolished DMSO-mediated Ald^R.

Conclusion: DMSO and Fenobam protect against Aβ toxicity through modulation of NT.

Keywords

C. elegans, DMSO, Aβ, Acetylcholine, Glutamate, Aldicarb, Levamisole, *daf-16*, Alzheimer disease, Ca2+, Neurotransmission

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Introduction

The world population is aging at an alarming rate, with the number of people aged 60 years and older expected to rise from 841 million in 2013 to more than 2 billion in 2050.[1] This will be an enormous social and financial burden on the societies worldwide. Aging is already a risk factor for neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinsons.[2] In AD, abnormal amyloid beta peptide (Aβ42) in the brain is known to cause severe neurotransmitter-signaling dysfunction, manifested as progressive dementia, loss of memory, and associated neurological complications in affected people.[3] One of the earliest findings regarding AD in the brain is loss of the major excitatory neurotransmitter, acetylcholine (ACh)-based

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cholinergic system. Currently, there is no cure for AD but only treatment with the inhibitor for the enzyme ACh esterase, which breaks down ACh at the synapse,[4] dopenil/galantimine.[5] Another drug being used is memantine, an antagonist of the major excitatory neurotransmitter glutamate's ionotropic N-Methyl-D-Aspartate (NMDA) receptor, which is important for learning and memory formation in the hippocampus,[6] for moderate to severe AD to manage daily life. [7] But both these drugs are of limited use. Despite decades of exhaustive research, not much is there on the horizon.[8] A better drug to delay or cure AD is an immediate necessity.

In general, neurotransmitters are synthesized and loaded into synaptic vesicles and taken to the synapse where they are released upon active electric impulse, especially depolarization. Ca^{2+} ions are essential for the synaptic release of neurotransmitters[9,10] including the two major excitatory neurotransmitters, ACh and glutamate (Glu), and their signaling system that are implicated in AD. Once released at the synapse, the neurotransmitter is quickly either sequestered back by transporters or broken down enzymatically (AChE) as in the case of ACh.[11] The released neurotransmitters bind to ionotropic/G-protein-coupled metabotropic receptors, which are multimeric consisting of heteromeric subunits and subtypes, in the postsynaptic neuron(s) or in the neuromuscular junction (NMJ) to bring about a multitude of outputs including movement and other behavioral responses. In the muscles, Ca^{2+} is required to bring about movement. Together, they form the exceptionally complex nervous system that is hard to decipher in organisms like mice and humans. Further, this complexity provides multiple drug targets to restore a semblance of normalcy in neurological and neurodegenerative diseases. More importantly, the fundamental mechanism of neurotransmission (NT) and neurotransmitters is conserved even in the easily amenable and simple model organism, *Caenorhabditis elegans* (*C. elagans*), with just 302 neurons unlike the billions in humans, making it a suitable platform for rapid preclinical drug screening.[12]

CL4176 is an established amyloid beta (Aβ) toxicity *C. elegans* worm model, in which AD causing human Aβ 1 to 42 is expressed in the muscle cells through myo-3 promoter. More importantly, in CL4176 worms, Aβ expression is brought about in the muscles by a temperature upshift, which employs a temperature-sensitive mutation in the mRNA surveillance system. This temperature-inducible muscle expression of Aβ results in a paralysis phenotype upon temperature upshift that is reproducible.[13] This forms an ideal preclinical model to screen the ACh and Glu neurotransmitter-signaling-related compounds—inhibitors, agonists and antagonists[14]—for abolition of Aβ toxicity. Additionally, as the solvents that solubilize the compounds could themselves be modulating Ach[15]/Glu-NT,[16] their contribution needs to be taken into consideration. Further, the mechanism of action of these solvents could again be addressed in the *C. elegans* system,[15] which will be a great advantage to the billion-dollar drug discovery industry.

Hence, here we address ways to alleviate AD causing Aβ toxicity-induced paralysis in CL4176 worms with (a) excitatory neurotransmitters ACh and Glu signaling related compounds, (b) the widely used solvent dimethyl sulfoxide (DMSO), (c) and the potential key components necessary to provide the protection.

Materials and Methods

Strains Used

Bristol N2: Wild-type, CL4176: *smg-1* (cc456); dvIs27 (myo-3p::Aβ [1–42]::let-851 3'UTR (UnTranslated Region)] + rol-6 [su1006]) X were from Caenorhabditis Genetics Centre, Minnesota, USA. GR1307: *daf-16* (mgDf50) was a gift from Arnab Mukhopadhyay from the National Institute of Immunology, New Delhi. All the *C. elegan*s strains except CL4176 were grown on Nematode Growth Medium (NGM) plates with *E. coli* (OP50) lawn as food at 20°C, following standard protocols.[17] CL4176 worms were maintained at 15°C.[18]

Chemicals

Dimethyl sulfoxide (SRL, India), Aldicarb (Ach esterase inhibitor;[19] Chemservice, USA), Levamisole- (agonist of *C. elegans* levamisole sensitive ionotropic Ach receptors-$iAChR^{ls}$[20,21]; Merck [formerly Sigma-Aldrich], PG9- maleate, increases Ach concentration at the synapse,[22] Ly341495 [GrpII mGluR antagonist],[23] Nefiracetam [nootrophic, agonist of GABA-A receptors],[24,25] Fenobam [negative allosteric modulator of GrpI mGluR-mGluR5], [26] Quisqualic acid-Glu agonist, which activates ionotrophic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/ Kainate glutamate receptors [iGluRs], and GrpI mGluRs)[27,28] were from TOCRIS Biosciences, a kind gift from Joseph H. Neale, Georgetown University, Washington DC, USA.

Preparation of Compound-containing Plates

Fenobam, quisqualic acid (QA), L-AP4, and PG-9 Maleate were prepared as 100 mM main stocks in DMSO. LY341495 was prepared as 25 mM stock in 1 M sodium hydroxide. All of these stocks were stored in a freezer at -80 ^{o}C and thawed when needed. Working stocks were prepared in M9 buffer. Compounds in M9 buffer at a volume of 200 ul were spread on four-cm OP50 *E. coli* lawn containing NGM plates.[19,29] The worms were transferred to these plates after half an hour. Dose response was carried out for all the compounds. The compounds Fenobam and QA, which were active at a final concentration of 10 uM, were chosen for further studies. During dose response, DMSO's protective effect was also noticed. Then, the DMSO combination with the drug Fenobam/QA was carried out. For this, 1 mM working stock of Fenobam/QA was prepared in DMSO and plated to a final concentration of 10 uM in M9 buffer.

Heat Induced Paralysis Assays

Synchronous population of worms were obtained by timed egg laying. For this, at least 10 to 15 adult CL4176 worms were passaged to each control (M9 buffer)/treatment plate and allowed to lay eggs for 4 to 6 h. The adult worms were then burned. The eggs were allowed to hatch into larva and were maintained at 16°C till L3 larval stage. The plates were then shifted to 25°C. The worms were scored as paralyzed or nonparalyzed from 36 h onwards.[18]

Aldicarb Assays

The standard aldicarb[19,30,31] assay was carried out with slight modifications. Aldicarb (0.1 mM) plates were prepared ½ h before the assay by spreading on the OP50 containing NGM agar plate. The L4 stage of the worms is considered as the age Day zero.[29,31,32] For these assays, on day one, adult worms were placed on assay plates and scored for paralysis over time, every 5 min, until all worms got paralyzed. The worms were considered paralyzed when they did not move in response to repeated prodding.

Levamisole Assays

Levamisole assays were carried out as per procedures.[19,20] NGM plates with 200µM levamisole were prepared half an hour before the assay. On day one, adult worms were used for assay. The scoring for paralysis was done at 30 min intervals and followed for 5 to 6 h.

Statistical Analysis

Graphs and statistical tests were carried out using Sigma Plot 10.0.

Results

Screening of Neurotransmitter Pathway Inhibitors/ Activators for Protection Against Aβ Toxicity

Various neurotransmitter pathway compounds—Glutamate-Fenobam, L-QA, L-AP4, and LY341495; ACh-PG-9 maleate and GABA-Nefiracetam—were screened at various doses for their ability to affect heat-induced AD causing Aβ expression in the muscle, mediated paralysis of CL4176 worms. Normally, CL4176 worms when shifted to 25 °C at L4 stage develop paralysis in 40 h to 44 h. Though not significant (Table 1), only Fenobam and L-QA were able to show delay (Figure 2A) or hasten (Figure 2B, Table 1) paralysis, respectively, compared to control. The rest of the compounds did not display any changes in the rate of paralysis in comparison with their controls (data not shown).

Table 1. Dominance of DMSO Over Glutamate Pathway Effects in CL4176 Worm

Treatment	Percentage Not Paralyzed at 64 h (Mean ± S.D)	*P*-Value
1% DMSO		
Control (M9 buffer)	6.30 ± 6.78	< .001
1% DMSO	54.00 ± 26.18	
2% DMSO		
Control (M9 buffer)	8.5 ± 5.06	< .001
2% DMSO	98.5 ± 3.0	
Fenobam		
Control (M9 buffer)	14.75 ± 20.28	
10 uM Fenobam	34.80 ± 26.35	Not significant
10 uM Fenobam + 1% DMSO	94.67 ± 4.16	.001
Quisqualic Acid		
Control (M9 buffer)	8.5±5.06	
10uM Quisqualic acid	12.75 ± 8.18	Not significant
10uM Quisqualic acid + 1% DMSO	43.5 ± 15.52	.005

Note: Number of worms are the same as in Figures 1 and 2.

High Concentration of DMSO Confers Protection Against Aβ Toxicity

All the aforementioned compounds were solubilized in DMSO (except LY341495, which was soluble in sodium hydroxide), and dose-response analysis of different DMSO concentrations such as 0.01%, 0.1%, 1%, and 2% DMSO on the heat–inducible paralysis assays of CL4176 worms was carried out. Of these, 1% DMSO and 2% DMSO treatment from embryo stage onwards showed significant delay in paralysis, 48% and 90%, respectively, after 64 h (Figure1A, B and Table 1) of temperature upshift compared to controls. Further, 2% DMSO-treated worms were not paralyzed even after 80 h of temperature upshift. Thus, 2% DMSO was the most protective against Aβ toxicity (Figure1B, Table 1).

Glutamate Reverses DMSO-mediated Protection

As the two Glu pathway compounds could marginally influence the rate of Aβ-induced paralysis, and the DMSO robustly delayed the paralysis, their combination was evaluated for further protection and to understand the mechanism of DMSO-mediated protection against Aβ toxicity. The presence of 1% DMSO along with Fenobam (10 uM), led to 95% unparalyzed worms versus 15% for control (M9 buffer) and 35% for Fenobam-alone treated CL4176 worms at the end of 64 h of temperature upshift to 25 °C.

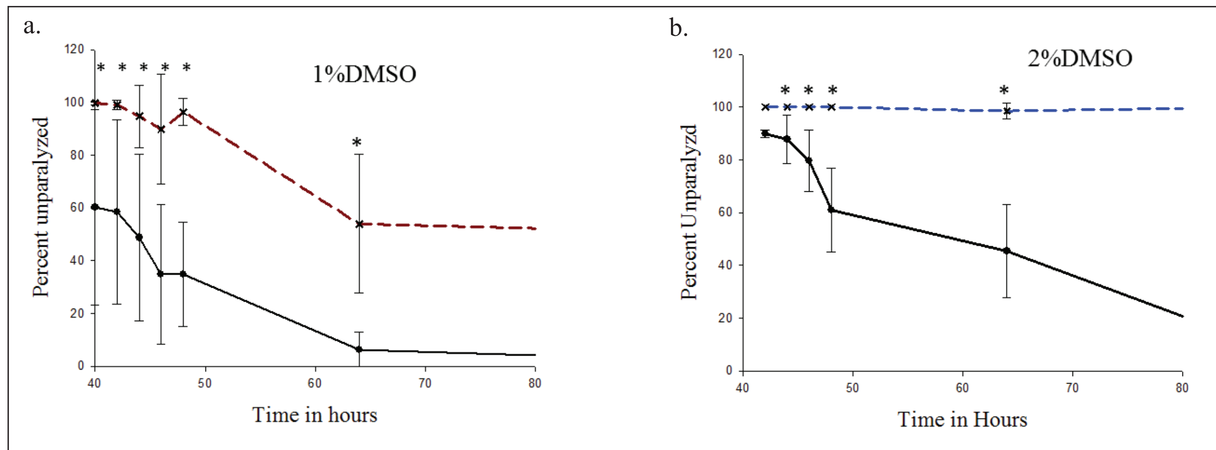

Figure 1. DMSO Delays Amyloid Beta Induced Paralysis

A. Control (Circle with Black Solid Line; *N* = 1663); 1% DMSO (Crosshair with Brown Dashes; *N* = 1605).

B. Control (Circle with Black Solid Line; *N* = 354); 2% DMSO (Crosshair with Blue Dashes *N* = 140). 2% DMSO Abolishes Paralysis Up to 80 h.

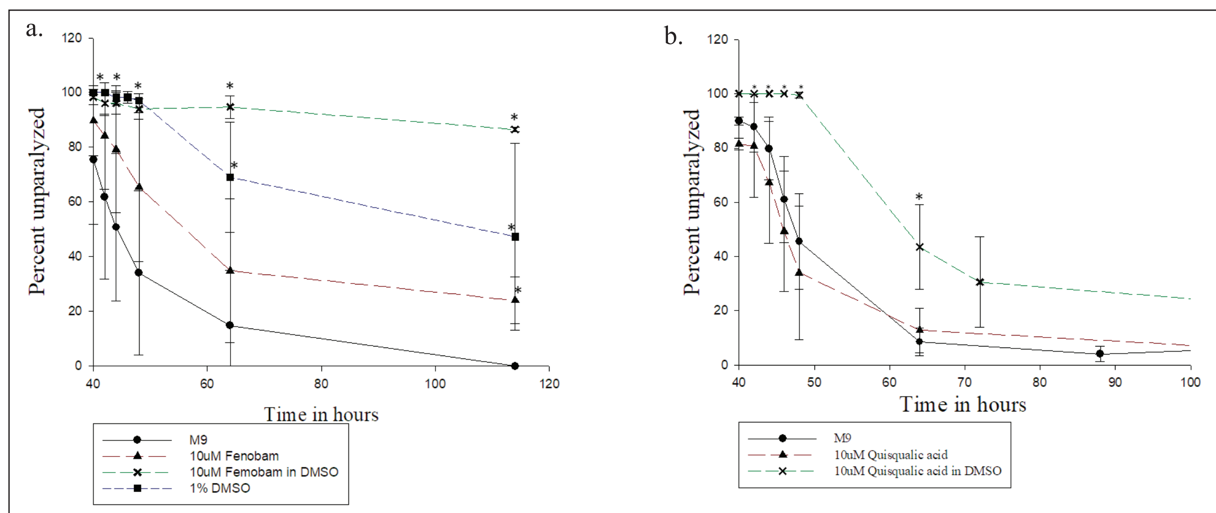

Figure 2. Glutamate-NT Reverses DMSO-mediated Protection

A. Control-M9 (*N* = 754); DMSO (1%; N = 449); Fenobam (10 uM; *N* = 446); Fenobam (10 uM) in 1% DMSO (*N* = 173).

B. Control (*N* = 354); Quisqualic Acid (10uM; *N* = 382); Quisqualic acid (10uM) in 1% DMSO (*N* = 219).

For Both A and B, Control (Black Circle with Black Solid Line); Fenobam (F)/Quisqualic Acid (QA; Black Triangle with Brown Long Dashes); DMSO (Black Square, Dark Blue dotted line) in Figure A is Common for Both Figure A and Figure B.

F/QA + 1% DMSO (Black Cross Hair with Blue Medium Dashes).

While Fenobam displayed a marginal delay of paralysis (20.5%), the presence of 1% DMSO increased it lot further (80%; Figure 2A, Table 1) compared to control (Table 1).

In case of QA and DMSO, the presence of DMSO could protect 43% versus 12.75% for QA alone while most of the control (M9) CL4176 worms were paralyzed at the end of 64 h (Figure 2B, Table 1). Protection of only 35% with QA and DMSO (1%) is providing strong evidence for a significant contribution of Glu-NT in Aβ-induced paralysis, thus, reinforcing that reduction of Glu-NT can enhance protection in AD worms.

DMSO Downregulates ACh Mediated Neurotransmission

As ACh is a major neurotransmitter implicated in AD, the influence of DMSO on ACh-NT was determined. The established aldicarb/levamisole paralysis assays were

performed. Aldicarb, an acetylcholine esterase inhibitor (AChEi), prevents the breakdown of ACh at the synapse/NMJ that leads to ACh accumulation at NMJ that eventually causes paralysis of the worms.[30,33] Levamisole acts as an agonist to the levamisole-sensitive ionotropic ACh receptors (iAChR^{ls}) of the muscle cells at the NMJ of *C. elegans*. Overstimulation of iAChR^{ls} with levamisole leads to continuous muscle contraction, resulting in paralysis.[20,34] These two together constitute an elegant strategy to measure pre and postsynaptic ACh-NT modulation at NMJ of *C. elegans*. As the temperature upshift of CL4176 worms will also lead to paralysis induced by Aβ toxicity, the aldicarb/levamisole assays were performed in them with chronic DMSO exposure but not temperature upshift. Two percent DMSO treatment was able to confer 39% and 87% delay in paralysis (Figure 3, Table 2) with aldicarb and levamisole, respectively, compared to controls. An increased delay in paralysis of 46% upon levamisole exposure in 2% DMSO-treated CL4176 worms suggests that though DMSO is exerting the protective effect against Aβ toxicity both pre and postsynaptically, it is more pronounced in postsynaptic ACh-NT from the muscles where Aβ too is expressed.

DMSO Modulates ACh-NT Differently in Wildtype N2 Worms

More Pronounced Aldicarb Resistance (Ald^R)

In wildtype N2 worms, DMSO acts quite differently in modulating ACh-NT compared to CL4176 worms. After 2 h of Ald exposure 1% DMSO-treated N2 worms were almost not paralyzed (99%) compared to 15% of the control worms (Figure 4A), unlike the CL4176 worms in which mean paralysis time was 57 min (Table 2) with all the worms paralyzed by 2 h (Figure 3A), that too with 2% DMSO exposure.

Levamisole Hypersensitivity

Unlike the CL4176 worms that showed levamisole resistance with DMSO exposure, 1% DMSO-treated N2 worms were hypersensitive to levamisole, and >80% of them were paralyzed (Figure 4B, Table 3) compared to 33% control, just 30 min after exposure to levamisole. This is providing a novel insight that the levamisole sensitive and insensitive iAChRs stoichiometry at the NMJ is probably altered in CL4176 worms compared to wildtype worms.

EDTA Can Reverse DMSO-induced Levamisole Hypersensitivity

Ca^{2+} is essential for NT, specifically in ACh-NT, for the release of neurotransmitters from the synaptic vesicles at the synapse/NMJ upon action potential generation.[9] Here, EDTA was used to sequester the Ca^{2+} ions.[35] Aldicarb assays on control; 1% DMSO, EDTA (0.45 mM,) and DMSO + EDTA treated worms revealed that while more than 80% control worms are paralyzed by 2 h, the other three groups of treated worms were largely unparalyzed (91% to 99%; Figure 4A). Though, the effect of DMSO is marginally reduced in EDTA combination, it still suggests that DMSO could be acting through either decreased Ca^{2+} release or some other pathway.

More importantly, EDTA (0.45 mM) was able to reverse the 1% DMSO-induced hypersensitivity (Figure 4B, Table 3)

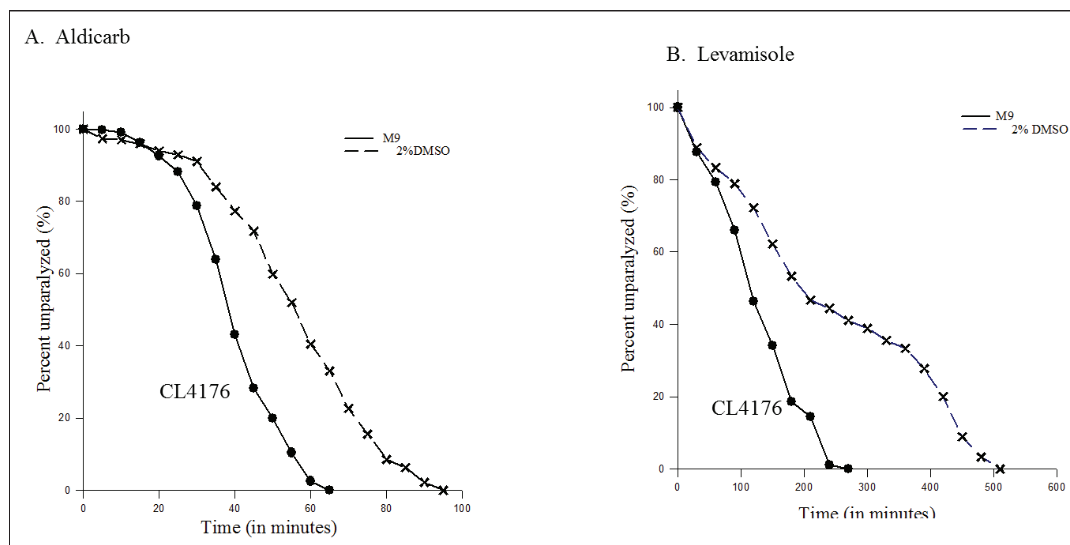

Figure 3. DMSO Downregulates Acetylcholine Neurotransmission

A. Aldicarb Resistance: Control (Solid Black Line, Circle; *N* = 356); 2% DMSO (Crosshair with Dashes; *N* = 271); *P* < .001

B. Levamisole Resistance: Control (Solid Black Line, Circle; *N* = 97); 2% DMSO (Dashes, Crosshair; *N* = 90); *P* < .001.

Table 2. DMSO Delays ACh Neurotransmission in CL4176 Worms

Treatment	Time Taken for Paralysis Mean ± S.D (Min)	*P* Value	% Delay in Paralysis
Aldicarb			
M9	41 ± 12	.001	39 (Ald^R)
2% DMSO	57 ± 20		
Levamisole			
M9	134 ± 66	.001	87 (lev^R)
2% DMSO	251 ± 158		

Note: Number of worms are same as in Figure 3.

Abbreviations: lev, Levamisole; Ald^R, Aldicarb resistance.

and restore to the level of control worms after 30 min of levamisole exposure (Figure 4B, Table 3). This effect tapers off as the time of levamisole exposure increases and follows the same pattern as control up to 120 min when almost all the worms are paralyzed in levamisole (Figure 4B, Table 3). Thus, 1% DMSO effect could be negated with EDTA as Ca^{2+} is essential for muscle contraction, and its depletion with EDTA could be negating DMSO effects.

The FOXO Transcription Factor Homolog, daf-16, is Essential for DMSO Effect

The aforementioned differences in aldicarb and levamisole sensitivities in the wildtype and CL4176 worms and its implications made us to explore for the downstream players.

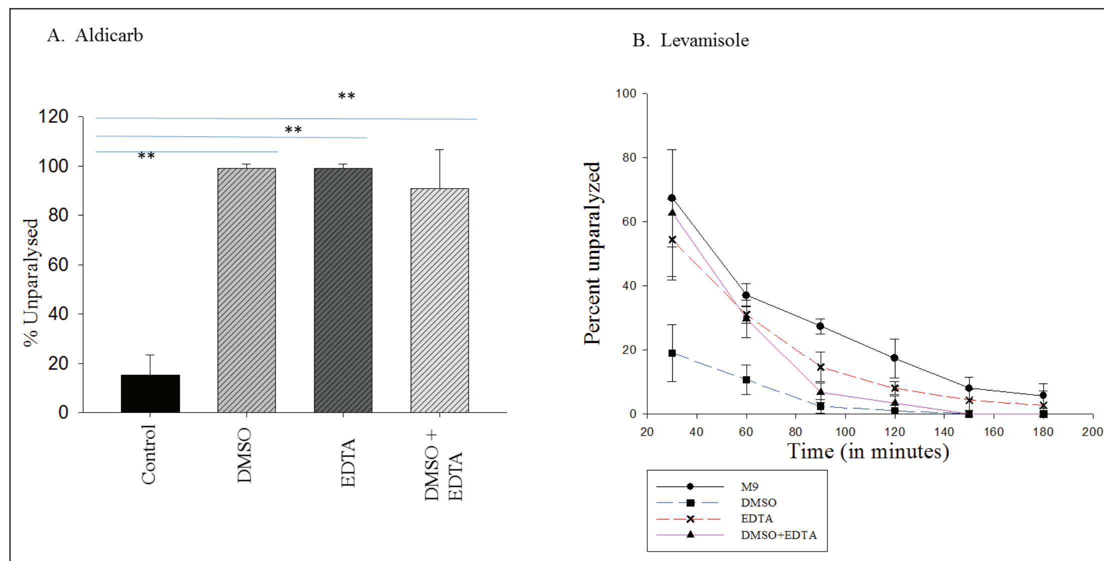

Figure 4. DMSO Acts Differently in Wildtype N2 Worms

A. Aldicarb Resistance: Control (Black; *N* = 225), DMSO (Grey; *N* = 230), EDTA (Dark Grey; *N* = 230), DMSO + EDTA (Light Grey; *N* = 230), $P < .001$

B. Levamisole Assay: Control (Black Solid Line, Circles; *N* = 130), DMSO (Blue Dashes with Square; *N* = 130), EDTA (Red Double Dash with Crosshair; *N* = 130) DMSO + EDTA (Pink Line with Triangle; *N* = 130). Statistics Given in Table 3.

Table 3. DMSO-mediated Levamisole Hypersensitivity Requires the Ca^{2+} Depleted by EDTA in Wildtype N2 Worms

Time	Group	Mean ± S.D (% Unparalyzed)	Comparison ($P < .05$)	*P*-Value (from One-way ANOVA)
30	C	67.33 ± 15.14		$P = .014$
	D	19 ± 8.82	C vs D	
	E	54.33 ± 12.67	D vs E	
	DE	62.67 ± 19.73	D vs DE	
60	C	37 ± 3.60		$P < .001$
	D	10.67 ± 4.61	C vs D; E vs D	
	E	31 ± 2.64		
	DE	29.67 ± 5.85	D vs DE	

(Table 3 continued)

(Table 3 continued)

Time	Group	Mean ± S.D (% Unparalyzed)	Comparison ($P < .05$)	P-Value (from One-way ANOVA)
90	C	27.3 ± 2.3		$P < .001$
	D	2.33 ± 2.08	C vs D;	
	E	14.67 ± 4.61	C vs E; D vs E; E vs DE	
	DE	6.67 ± 3.05	C vs DE;	
120	C	17.33 ± 6.11		$P = .002$
	D	1.0 ± 1.73	C vs D	
	E	8.0 ± 2.0	C vs E; D vs E;	
	DE	3.33 ± 2.30	C vs DE	

Note: Control, C; D, DMSO (1%); E, EDTA (0.45 mM); DE, DMSO (1%) + EDTA (0.45 mM); the total number of worms assayed for each treatment: $N = 130$. Cumulative of three independent experiments with $N = 40/45$ in each group.

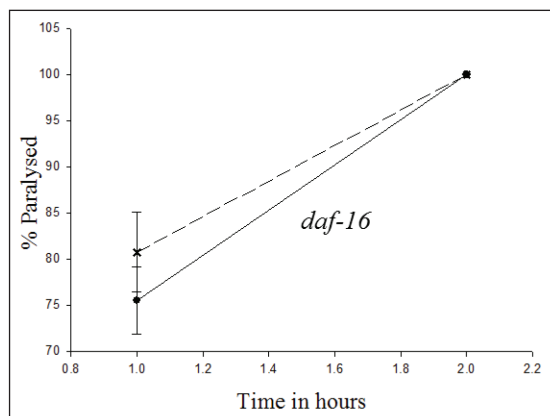

Figure 5. daf-16 is Essential for DMSO Effect
Control (Black Solid Line; $N = 80$); DMSO (1%; Dashed Line; $N = 86$).

Intriguingly, when an attempt was made to see if 1% DMSO was dependent on one of the versatile transcription factors, originally found to be the major downstream player in the reduced insulin/insulin-like growth factor signaling (IIS) pathway, FOXO transcription factor homolog, *daf-16*, with its *daf-16(mgDf50)* deletion mutant[36] using aldicarb assays, DMSO could not elicit its normal Ald^R that it conferred on the wildtype and CL4176 worms. Essentially, 1% DMSO-treated *daf-16* worms showed a similar aldicarb sensitivity pattern as the control, with all the worms getting paralyzed by 2 h, irrespective of DMSO exposure (Figure 5). This suggests that the protective effect of DMSO is dependent on *daf-16* and its induction of expression of wide array of downstream genes.

Discussion

As AD is the most prevalent neurodegenerative disease and an enormous social and financial liability, drugs to cure the disease is of great urgency and necessity. Further, it will provide a dignified life at old age. AD is caused because of the progressive neurotransmitter-signaling dysfunction caused because of abnormal Amyloid plaques by Aβ peptide. The two major excitatory neurotransmitters, Glu and ACh are involved in AD. As screening for drugs in higher animal models is time-consuming and expensive, we utilized the well-established preclinical model in *C. elegans*, CL4176.[37] Compounds that act on an array of neurotransmitter-related pathways were screened. They are as follows: Nefiracetam, a cognitive enhancer that raises the ACh levels in the cortical regions of rat brains[24]; PG-9 Maleate, an amnesia reversal drug, an ACh releaser in amnesia-induced mice and increased nerve growth factor synthesis in astrocytes[22]; L-AP4 (L-2-amino-4-phosphonobutyric acid), Grp III mGluR agonist without selectivity for subtypes[38] and a proposed drug to treat Parkinson's disease[39]; LY341495, a potent Class II mGluR antagonist[23]; Fenobam, an anxiolytic and a strong mGluR5 antagonist[26] proposed for autism treatment[40]; and L-QA, agonist of iGluR–AMPA/Kainate receptors and GrpI mGluRs.[27,28] Of these, only Fenobam and QA could either delay or accelerate Aβ-induced paralysis in the AD model worms, but are statistically not significant (Figure 2, Table 1).

In contrast, DMSO, a universal solvent that is frequently used to dissolve compounds for screening in biological systems and is also a cryoprotectant, which was used to solubilize most of the above compounds, showed significant protection against Aβ-induced paralysis at 1% DMSO, and 2% DMSO provided two fold better protection (Figure 1A, 1B; Table 1). DMSO at 1% is known to increase dendritic spine density, memory, and olfactory sensitivity and to attenuate anxiety in AD model mice.[16] Here, the inclusion of DMSO (1%) with Fenobam could increase the 1% DMSO or 10 uM Fenobam effect to that of 2% DMSO (Figure 2A), but not with QA (only ~50%), which essentially acts as a Glu agonist (Figure 2A, 2B Table 1). Thus, higher Glu action is dominant over 1% DMSO. More importantly, suppression of Glu-NT can alter protection against Aβ toxicity as iterated by NMDA receptor antagonist, memantine, being helpful against moderate and severe AD.[41] Given the exceptional complexity of Glu–NT signaling network, a combinatorial approach with

receptor-type specific Glu agonists and antagonists may give potential drugs to treat AD.

In AD, loss of the cholinergic system is very well known. Increase in ACh with Donepezil/galantamine,[5] the prescribed drug for AD, is shown to provide modest protection in AD patients. So, we determined how DMSO (2%) altered ACh-NT with the elegant aldicarb/levamisole paralysis assays in both AD model worms, CL4176, and wildtype-N2 worms. In CL4176, without induction of Aβ expression through temperature, upshift aldicarb and levamisole resistance (Figure 3a, 3b, Table 2) was noticed. But the temperature upshift abolished Lev^R (data not shown). This is an indication that though DMSO can alter ACh-NT, Aβ expression abrogates it in the AD model worms, suggesting that DMSO is not protecting through direct alteration of ACh-NT. On the contrary, in the wildtype N2 worms, 1% DMSO treatment induced levamisole hypersensitivity (Figure 4B). But in wildtype worms, Ald^R (Figure 4A) was retained and more pronounced with a 40% increase. This is suggesting novel insights into the alteration of overall ACh-NT, and specifically that ionotropic ACh receptor (iAChR) stoichiometry in the AD model worms could be obtained with a detailed study, though it may be challenging. There is a possibility of marginal expression of Aβ even without temperature upshift, and it is known that Aβ directly interacts with α7 type iAChR, which are the levamisole insensitive receptors in *C. elegans.*[42] Thus, DMSO can alter ACh-NT to provide Aβ toxicity protection in CL4176 worms, but not directly. Identification of the iAChR, both levamisole sensitive and insensitive and their interactors, localization in NMJ and overall turnover can provide insights into Aβ toxicity and provide novel drug targets to treat AD.

So, we went on to identify the potential mechanism of DMSO action through use of EDTA, the known Ca^{2+} ion chelator, as Ca^{2+} is required presynaptically for neurotransmitter release and at the muscles for contraction. Contrary to the reports of DMSO increasing Ca^{2+} release,[43] we find DMSO to induce to Ald^R (Figure 3A and 4A). As expected, EDTA did induce aldicarb and levamisole resistance in the WT worms. Presynaptically, both DMSO and EDTA induced Ald^R either alone or together (Figure 4A), indicating they could alter ACh release through the same or different means. Postsynaptically in the NMJ-mediated muscle contraction, EDTA caused levamisole resistance and could reverse DMSO-induced levamisole hypersensitivity (Figure 4B, Table 3) up to 90 min. Thus, postsynaptic alteration by DMSO seems to be through increasing Ca^{2+} permeability, which is in line with the reports of DMSO increasing Ca^{2+} permeability.

Unexpectedly, 1% DMSO requires the presence of the FOXO transcription factor homolog, *daf-16*, to mount Ald^R (Figure 5) as evidenced in the deletion mutant of *daf-16* (*mgDf50*) in which the coding region is completely eliminated.[36] Neuronal *daf-16* is shown to modulate both ACh[44] and Glu-NT.[45] It alters Glu-NT through change in the expression of glutaminase. Further, *daf-16* is interconnected with several pathways that include an insulin-signaling pathway, Target of Rapamycin (TOR), Adenosine mono phosphate-activated protein kinase (AMPK), and germline signaling. Many of these pathways are involved in regulation of lifespan, metabolism, and various cellular processes (reviewed in Sun et al., 2017).[44] For thermotolerance, *daf-16* is dependent on ACh signaling.[46] Similarly, Glu signaling is required by *daf-16* to relay longevity signals to the intestine in *C. elegans* in response to heat and cold.[47] Further evaluation of various mutants of *daf-16* and a transgenic rescue of *daf-16* with its own promoter or tissue-specific promoters will provide insights on how *daf-16* alters ACh-NT when treated with DMSO. As *daf-16* is known to turn on a multitude of genes, global/neuronal gene expression analysis after chronic DMSO exposure will provide novel insights into nervous system structure and function. In addition to *daf-16*, other transcription factors could also play important role in DMSO effect. They should be evaluated again to identify their contribution in DMSO-mediated ACh-NT modulation.

Even more important is the wide use of DMSO as a solvent of choice in the billion-dollar drug discovery research carried out in both academia and industry. In addition, DMSO is widely used in the paint industry, herbicides, and also in the environment.[48] Hence, DMSO-induced global gene expression and functional changes need to be taken into serious consideration when DMSO is chosen as a solvent. DMSO at a lower concentration to treat AD can be considered with caution as it does provide protection in the AD model mice.[16] So far, DMSO is reported to be used to treat amyloidosis in human patients with rheumatoid arthritis [49] and renal failure.[50] In both these cases, it was able to alleviate only secondary amyloidosis. Further, because of its ready permeability through any cell type, including the skin in animals and humans,[48] extreme caution is required in DMSO usage. DMSO is known to cause nonspecific adverse effects, like apoptosis induction in developing CNS of rats,[51] cytotoxic effects in cochlear organotypic cultures[52] and mouse embryos,[53] and renal failure[48] beyond concentrations of 1%.

Overall, modulation of ACh-NT as seen with reserpine[19,54,55], an antihypertensive drug, Glu-NT, and Ca^{2+} ions can delay Aβ-induced paralysis, and suppression of Glu-NT can greatly increase ACh-NT in *C. elegans*.[56] Interestingly, the FOXO transcription factor, *daf-16,* which is suppressed in the insulin/insulin-like growth factor signaling, is required for ACh-NT. Together, these open up a multipronged direction to new drug discovery strategy to prevent AD.

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