Neuroscience Letters 439 (2008) 129–133

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Acetaminophen attenuates dopamine neuron degeneration in animal models of Parkinson's disease

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ARTICLE INFO

Article history:
Received 13 February 2008
Received in revised form 19 April 2008
Accepted 2 May 2008

Keywords:
Neuroprotection
6-OHDA
α-Synuclein
Parkinson's disease
C. elegans

ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disorder with ~2% of people over age 65 suffering from this disease. Risk factors for PD involve interplay between still poorly defined genetic and non-genetic contributors, but appear to converge upon cellular pathways that mediate protein misfolding and oxidative stress that lead to dopaminergic neuron loss. The identification of either new or repurposed drugs that exhibit benefit in slowing the age-dependent neuronal damage that occurs in PD is a significant goal of much ongoing research. We have exploited the nematode *Caenorhabditis elegans* as a model system by which the neuroprotective capacity of acetaminophen could be rapidly evaluated for efficacy in attenuating dopamine (DA) neurodegeneration. Using three independent and established neurodegenerative models in *C. elegans*, we assayed for acetaminophen-dependent rescue in response to: (1) over-expression of the PD-associated protein, α-synuclein; (2) acute exposure to 6-hydroxydopamine (6-OHDA); (3) excess intracellular DA production due to over-expression of the DA biosynthetic enzyme, tyrosine hydroxylase (TH). These data suggest that acetaminophen significantly protected *C. elegans* DA neurons from stressors related to oxidative damage, but not protein misfolding. Taken together, these studies imply an activity for acetaminophen in the attenuation of DA neuron loss that, following essential corroborative analyses in mammalian systems, may represent a potential benefit for PD.

The most logical route toward therapeutic intervention for neurodegenerative diseases involves the identification of small molecules that have the ability to provide neuroprotection. Limited animal model studies have shown that acetaminophen might protect neurons from degeneration. For example, acetaminophen can protect primary rat embryonic DA neurons from glutamate toxicity [6]. This is an important finding for the treatment of PD as DA neurons, which selectively degenerate in this disease, are particularly vulnerable to glutamate toxicity *in vitro* [15]. Additionally, acetaminophen provided partial neuroprotection in rats treated with 1-methyl-4-phenyl pyridinium (MPP^+), a neurotoxin that induces DA neurodegeneration [17]. These results suggest that acetaminophen could be a prophylactic, as well as adjuvant, therapy for neurodegenerative diseases such as PD. However, the mechanism behind acetaminophen-induced neuroprotection is still not known.

DA neuron loss is a hallmark of PD. Several distinct mechanisms have been associated with DA neuron decline. Some of these involve genetic mutations and others are a result of environmental exposures. Our laboratory has been examining the relationship between cellular stress and its functional consequences for PD using the nematode animal model system, *C. elegans* [5]. Despite its evolutionary distance from humans, *C. elegans* neurons retain many aspects of mammalian neuronal function including ion channels, neurotransmitters (dopamine, serotonin, acetylcholine, GABA, etc.), vesicular transporters, receptors, and synaptic components [2,7]. In this regard, we have developed several DA neurodegeneration models whereby there is a decline in DA neuron survival over the aging process; these models display degenerative phenotypes from a dominant genetic cause of PD, a toxin, or excess intracellular DA production.

The greatest strength of *C. elegans* as a model system is its ability to be used for screening purposes—be those genetic, genomic or chemical. There is a growing body of literature showing the utility of using *C. elegans* for pharmacological research. A variety of compounds ranging from drugs associated with modulating neurotransmitter activity [1,18] to epilepsy modifiers [10,25] have been successfully employed in this nematode. Here, we exploited the advantages of our DA neuron stress assays to determine which cellular malfunctions can be assuaged by acetaminophen exposure.

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doi:10.1016/j.neulet.2008.05.003

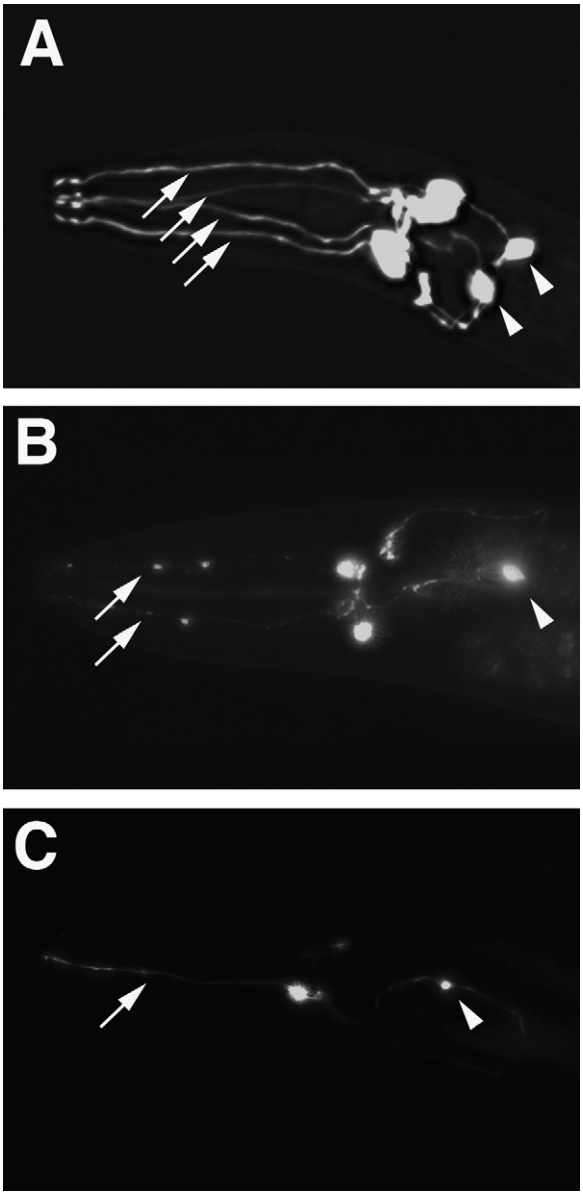

Fig. 1. *C. elegans* DA neurons visualized with GFP. (A) Worms retain all six anterior DA neurons without toxic insult (genetic or environmental); arrows depict the four CEP neuron processes and arrowheads indicate the ADE cell bodies in an adult worm. (B) Neurons within worms exposed to 6-OHDA undergo acute degenerative changes. This worm exhibits only 2 of 4 CEP neurons and 1 of 2 ADE neurons (arrowhead); the remaining 2 CEP neurons display dendrite blebbing and cell body rounding (arrows). (C) Most worms expressing α-synuclein within the DA neurons are missing anterior DA neurons when they are 7-day-old adults; only 1 of 4 CEP (arrow) and 1 of 2 ADE (arrowhead) DA neurons are present.

Nematodes were maintained using standard procedures [4]. Acetaminophen (Sigma A7085) was dissolved in molten nematode growth medium (NGM) before pouring in Petri dishes. The following final concentrations of acetaminophen were tested: 0.1, 1, 2, and 9.2 mM. Occasionally 10× higher concentrations of compounds are needed in worm assays as the worm cuticle can impede the uptake of compounds [20].

Compared with the ~100 billion neurons of the human brain, *C. elegans* hermaphrodites have exactly 302 neurons, precisely eight of which produce DA. Six of the DA neurons are located within the anterior-most region of the animal, consisting of two pairs of CEP (cephalic) and one pair of ADE (anterior deirid) neurons (Fig. 1A). For the ease of analyses, these six neurons were monitored in our assays; in all cases transgenic worms expressed GFP exclusively within the DA neurons ($P_{dat\text{-}1}$::GFP) [19]. To visualize these neurons, worms were mounted onto 2% agarose pads and immobilized with 3 mM levamisole before examination with a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP HYQ filter cube (Chroma). A neuron was scored as "wild-type" when its cell body and process (the prominent dendrites in CEP neurons or axons in ADE neurons) were intact. A neuron was scored as degenerating when at least one degenerative change such as "dendrite blebbing" (Fig. 1B), "cell body rounding" (Fig. 1B), or "cell body and/or process loss" (Fig. 1C) was observed. All six anterior DA neurons were scored in each animal. An unequivocal advantage of *C. elegans* is that detailed quantitative analyses of individual cells are achievable.

Images were captured with a Cool Snap HQ CCD camera (Photometrics) driven by MetaMorph Software (Universal Imaging). Statistics were performed by direct comparison between each treatment/control pair using the Fisher Exact Test (http://www.langsrud.com/fisher.htm).

Recent genetic discoveries have implicated specific proteins, such as α-synuclein, in the pathogenesis of PD. Genomic multiplication of the wild-type α-synuclein gene results in PD, indicating that over-expression of this protein alone can lead to the disease [11,21], We have established that over-expression of wild-type human α-synuclein under control of $P_{dat\text{-}1}$ promoter results in age- and dose-dependent neurodegeneration in *C. elegans* [5,13]. Worm neurons do not display high levels of degeneration until they are adults. Since DA neurodegeneration is slow in these animals (occurs over the course of a week), the complete loss of one or more DA neurons is the most commonly observed phenotype (Fig. 1C). These isogenic worms express $P_{dat\text{-}1}$::GFP and $P_{dat\text{-}1}$::α-synuclein (strain UA18 (*baEx18*)) [5]; herein they will be referred to simply as α-synuclein-expressing worms. Prior confirmation of human α-synuclein expression within the DA neurons of this transgenic strain was performed using both semi-quantitative RT-PCR [13] and immunolocalization [5]. Previously these same animals have been used to validate the neuroprotective capacity of specific gene products, such as torsinA, a protein with chaperone activity, and several Rab GTPases, which are involved in ER to Golgi transport [5,8,12].

To examine the effect of acetaminophen on α-synuclein-induced neurodegeneration, approximately 20 gravid adults (P_0 generation) were placed on NGM plates with either acetaminophen or solvent (water). When the F_1 larvae reached the L4 stage, ~500–1000 worms were then transferred onto 5–10 NGM plates, prepared with or without acetaminophen and 5-fluoro-2-deoxyuridine (FUDR; Fisher Scientific) at a concentration of 0.04 mg/mL media to prevent the development of offspring. When the F_1 animals aged to day 4 of adulthood, between 50 and 100 F_1 worms from each acetaminophen concentration were assayed for degenerating DA neurons using the criteria described above. Since the six anterior DA neurons were examined from each worm, and the analyses were repeated between two and four times per condition, with 25 worms/replicate, this corresponded to 300–600 neurons analyzed per concentration.

Acetaminophen was not significantly neuroprotective against α-synuclein-induced neurodegeneration at any concentration tested (Fig. 2). When solvent (water) was added to the media, 33.4% of the neurons from 4-day adult worms displayed degenerative changes while 35.5%, 31.7%, 29.7%, and 34.7% of the neurons were degenerating when worms were treated with 0.1 mM, 1 mM, 2 mM, and 9.2 mM acetaminophen, respectively.

Exposure to 6-OHDA causes formation of reactive oxygen species (ROS) and subsequently leads to DA neuron death via an undefined apoptotic mechanism. Worm DA neurons undergo a

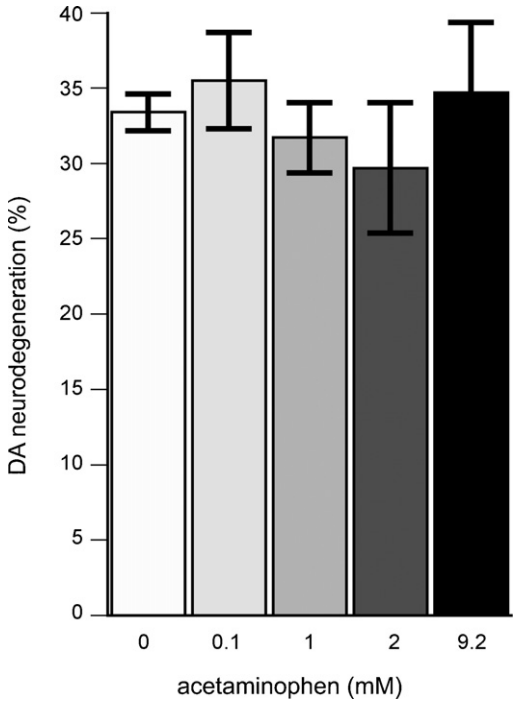

Fig. 2. Acetaminophen does not protect *C. elegans* DA neurons from degeneration resulting from over-expression of α-synuclein at any concentration tested when compared to animals exposed to the solvent (water) only. Approximately 300 neurons from 100 worms were examined at each concentration.

reproducible death from exposure to 10 mM 6-OHDA that begins within ~6 h of treatment [5,19]. These acute changes include readily observable CEP dendrite blebbing and cell body rounding (Fig. 1B). DA neurodegenerative changes observed at later time points (i.e., 48 and 72 h) typically involve complete loss of neurons.

We examined the impact of acetaminophen on the cellular effects caused by 6-OHDA. Age-synchronized $P_{dat\text{-}1}$::GFP worms [19] were obtained by treating gravid adults with 2% sodium hypochlorite, 0.5 M NaOH to isolate embryos [16]. These embryos were grown for 30 h at 25 °C in the presence of various concentrations of acetaminophen. At the L3-stage, larvae were incubated with 10 mM 6-OHDA (Sigma) in 2 mM ascorbic acid for 1 h with gentle agitation every 10 min [19]. The worms were then washed and spread onto NGM plates seeded with bacteria (OP50) and additional acetaminophen (at the same concentrations used before 6-OHDA treatment). Immediately after 6-OHDA treatment, worms containing the transgene were selected under a fluorescence dissecting microscope, based on the presence of GFP, and transferred to a fresh NGM plate seeded with OP50 (with or without acetaminophen). Worms were scored at time points ranging from 6 to 72 h post-6-OHDA exposure. After analysis at the 24-h time point, worms were then transferred to 0.04 mg FUDR/mL plates to reduce production of progeny, with the appropriate concentration of acetaminophen, for analysis of the 48 and 72-h time points. For each time point, neurons from 50 to 60 worms were analyzed, for a total of 300–360 neurons scored. The loss of DA neurons in worms is not lethal to these animals and results in only subtle behavioral deficits.

We determined that low concentrations of acetaminophen significantly prevented DA neurodegeneration when compared to solvent controls (white bars), as shown in Fig. 3 ($P < 0.05$). Notably, a concentration of 0.1 mM acetaminophen protected DA neurons at all time points assayed while 1 mM acetaminophen only protected DA neurons at early time points. Higher concentrations of acetaminophen (2 and 9.2 mM) did not protect DA neurons from 6-OHDA-induced degeneration at any time point assayed.

We have established an *in vivo* model to study the effect of altered DA metabolism on neurodegeneration [5]. To increase intraneuronal DA production *in vivo*, we over-expressed the *C. elegans cat-2* gene, encoding the nematode homolog of the DA biosynthetic enzyme, TH. Over-expression of the CAT-2 protein in DA neurons causes degeneration at all developmental stages but becomes more pronounced as animals age. For example, when these animals are 5 days old, 47.9% of the neurons are wild-type, but by the time they are 7 days old, only 23.3% of the neuron population displays wild-type DA neurons [5]. Changes in these neurons are phenotypically similar to those observed in α-synuclein-expressing animals. That is, the neurons are either present or absent. We rarely observe intermediate degenerative changes, such as dendrite blebbing, in these animals. We assayed the influence of acetaminophen on neurodegeneration in this model using worms expressing $P_{dat\text{-}1}$::GFP and $P_{dat\text{-}1}$::CAT-2 (strain UA23 (*baEx23*)) [5]. The worm breeding and exposure protocols were identical to those described for α-synuclein above.

We determined that all concentrations of acetaminophen tested were able to significantly suppress TH-induced DA neurodegen-

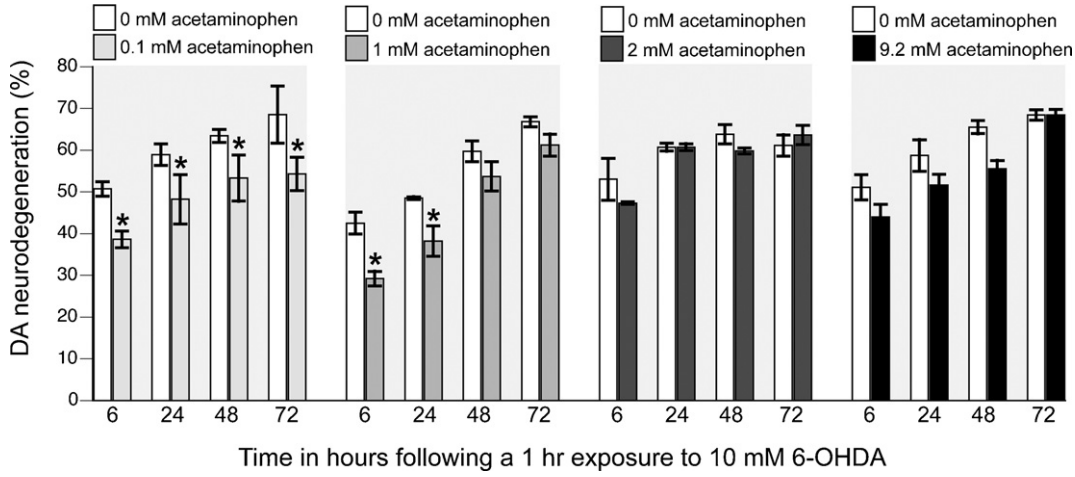

Fig. 3. Low concentrations of acetaminophen protect *C. elegans* DA neurons from short-term 6-OHDA exposure. The lowest concentration of acetaminophen tested, 0.1 mM, significantly protected DA neurons at all times assayed while 1.0 mM acetaminophen protected DA neurons at the 6 and 24 h time points ($P < 0.05$). Approximately 300 neurons from 50 worms were scored at each concentration and at each time point.

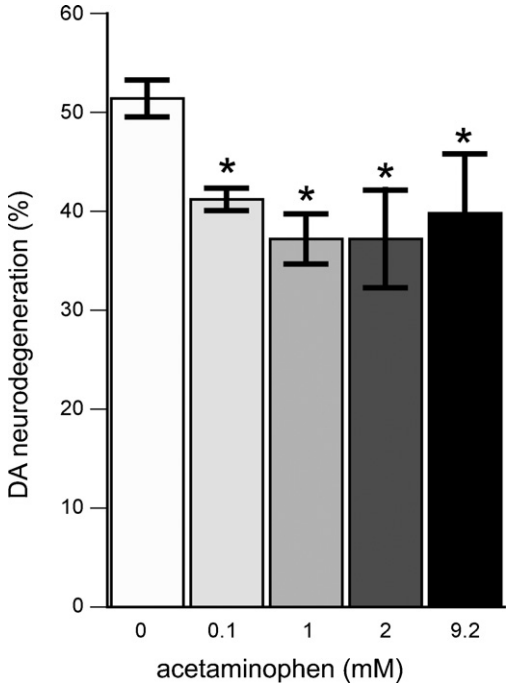

Fig. 4. Acetaminophen exposure protects *C. elegans* DA neurons from excess TH via over-expression of the *cat-2* gene. Significant protection was observed for all concentrations of acetaminophen examined ($P < 0.05$). Approximately 300 neurons from 100 5-day-old worms were examined for each concentration.

eration at day 5 of development ($P < 0.05$) (Fig. 4). Specifically, when worms were treated with 0.1 mM, 1 mM, 2 mM, and 9.2 mM acetaminophen, 41.2%, 37.2%, 37.2%, and 39.8% of the neurons were scored as degenerating, respectively, compared to 51.4% of the neurons exposed to solvent (water) only.

Our data reveal that acetaminophen demonstrated significant neuroprotective activity in specific scenarios of DA neurodegeneration in *C. elegans*. The different nematode models of DA neurodegeneration utilized in this study enabled us to infer potential mechanistic differences by which acetaminophen may exert its function. DA neurons are particularly susceptible to oxidative stress as a result of DA metabolism, as well as the presence of other intracellular factors favoring the formation of ROS [3]. Single nucleotide polymorphisms in specific genes encoding enzymes in the DA metabolic pathway can also increase susceptibility to PD [22]. In this context, the degeneration observed in animals over-expressing TH presumably correlates to oxidation of excess intracellular DA. Similarly, the neurodegeneration induced by 6-OHDA is an established result of *in vivo* oxidative stress caused by this hydroxylated form of DA.

Interestingly, while acetaminophen treatment did not enhance neuroprotection of DA neurons in animals over-expressing human α-synuclein, these same neurons received significant benefit from such treatment in worms over-expressing TH or exposed to 6-OHDA. This suggests that the beneficial effect to DA neuron survival observed with acetaminophen is not the outcome of any effect on protein misfolding, but is likely due to a role of this molecule in limiting oxidative stress. It should be noted that acetaminophen inhibited the formation of α-synuclein fibrils, as well as destabilized preformed fibrils, *in vitro* [14]. Given the disputed role for aggregated species of α-synuclein as being either cytotoxic or protective [24], it is possible that the net effect of acetaminophen on α-synuclein *in vivo* is neutral.

Familial PD accounts for only 5–10% of known cases. However, several of the genes linked to PD are predicted to function in cellular pathways involving the management of protein degradation and oxidative stress [9]. Thus, maintaining a cytological threshold against harmful environmental influences is central to disease etiology. Sporadic PD is traditionally thought to be a late-onset disorder associated with toxic exposures on the background of genetically susceptible individuals. Moreover, the cumulative effects of aging clearly are an unequivocal risk factor for PD and may be linked to the eventual failure of naturally protective cellular systems that erode over time [23]. Further investigation of the neuroprotective activity of acetaminophen in mammalian systems is warranted, given the prospect for this common medication to be used for prophylactic, as well as adjuvant, therapy for neurodegenerative diseases resulting from oxidative damage.

Acknowledgements

We wish to acknowledge the cooperative spirit of all Caldwell lab members. This work was supported by a grant from McNeil Consumer and Specialty Pharmaceuticals to KAC.

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