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Uridine Promotes Neurite Outgrowth in Neuro2a Cells

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Abstract

Neurodegenerative diseases such as Alzheimer's and Parkinson's are the main causes of age-related dementia. These diseases can be due to neuronal cell death and/or impairment of neurite outgrowth. Giant oyster mushroom (GOM), *Pleurotus giganteus*, is used as a nootropic to improve cognitive function. GOM can also be used to prevent the onset of dementia. The underlying mechanism behind the medicinal property of GOM is unclear. Previous studies have shown that GOM has a high concentration of uridine. In this study, I examined the effects of uridine on neurite outgrowth in the Neuro-2a (N2a) neuroblastoma cell line. We also examined the effects of various concentrations of uridine on neurite outgrowth in N2a cells. When exposed to uridine, N2a cells produced significantly longer neurite extensions ($p<0.001$) and exhibited a significant increase in neurite-bearing cells ($p<0.001$) with an ideal concentration of 100 μM. Our results suggest that uridine significantly promoted neurite outgrowth in N2a cells ($p<0.001$). Future studies are required to identify the mechanism behind uridines therapeutic potential on neurodegenerative diseases.

Background

Neurodegenerative diseases have major social and economic burdens on the world's increasing population. The number of people living with dementia-related illnesses, such as Alzheimer's and Parkinson's disease, is continuing to increase at a rapid rate. A large portion of the geriatric population will experience some form of dementia-related illnesses, increasing the economic burden of heath care. With the central nervous system's (CNS) inability to repair itself after injury or progressive disease damage, there is currently no treatment for recovering human nerve function. The majority of therapeutic medicine found to prevent neurodegeneration are unable to cross the blood-brain barrier and reach the CNS [1,2]. With the increasing need to prevent, or perhaps impede, the development and debilitating effects of neurodegenerative diseases, many people are turning to a more wholistic approach.

One approach to this issue is to be aware of age-related diseases and to take action towards their prevention through dietary supplements and functional foods. An increasing amount of research has been focusing on functional foods and their bioactive

constituents in our everyday diet [8]. Mushrooms have become known for their health benefits beyond providing nutrients. With an entire kingdom containing unique secondary metabolites, some can be used as an unlimited source of new pharmaceutical products [3]. Culinary-medicinal mushrooms have been used for their bioactive, secondary metabolites to reduce beta amyloid-induced neurotoxicity, neurite outgrowth stimulation, nerve growth factor synthesis, neuroprotective, antioxidant and anti-inflammatory effects [4]. Vitamin D-enriched white button mushrooms (*Agaricus bisporus*) have been shown to improve the memory in mice for Alzheimer's diesease [5]. Mushrooms have even been found to produce compounds that promote neurite outgrowth. One example are erinacines and hericenones, isolated from the mushroom *Hericium erinaceus*, commonly called lions main, for their neuroprotective properties [6]. Since these brain-improving compounds can only be isolated from the mushrooms that produce them, an increasing amount of research is needed in order to identify the mechanism behind their medicinal properties.

Pleurotus giganteus, commonly known as giant oyster mushroom (GOM), is used for culinary purposes. The consumption of this mushroom goes as far back as the indigenous people of Peninsular Malaysia [7]. Mushrooms of *Pleurotus* are commonly consumed all over the world for their flavor and high nutritional value [8]. Many *in vitro* studies of GOM have evaluated its anticancer, antioxidative, antifungal, hepatoprotective and neurite outgrowth capabilities [3-6,9-12]. GOM extracts exhibited neurite outgrowth in rat pheochromocytoma (PC12) and Neuro-2a (N2a) neuroblastoma cells [13,14]. The main bioactive ingredient of GOM is believed to be uridine.

Uridine is an RNA nucleotide and has been identified in multiple different mushroom species [15]. Specifically, it was recognized as one of the main bioactive compounds in the medicinal mushroom, *Cordyceps militaris*, containing 45.4 mg/kg extract of uridine [16]. Uridine is also present in breast milk [17] and, as a nucleotide, it is reported to have important physiological roles in breast feeding infants [18]. Uridine contributes to brain phosphatidylcholine synthesis via the Kennedy pathway [19, 20]. Its uptake into the brain and through the blood brain barrier (BBB) is initiated by specific nucleotide transporters. The rate at which uptake occurs is a major factor determining phosphatide synthesis. With uridine being a precursor of phosphatidylcholine, a

membrane constituent, its presence in GOM may give us a better understanding of its medicinal properties. Previous studies have shown GOM extract increases neuronal outgrowth with uridine being the active ingredient [12].

In this study, we examined the effects of uridine on neurite outgrowth in Neuro-2a (N2a) neuroblastoma cell line. We found that treatment of N2a cultures with uridine significantly increased neurite extension, combined length of neurites per cell, as well as the percent of neurite-bearing cells.

Methods

Materials and cell culturing

Mouse neuroblastoma cells (N2a) were obtained from American Type Culture Collection (Manassas, VA). The N2a cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), with L-glutamine, penicillin-streptomycin (Thermo-Fisher Scientific) and fetal bovine serum (FBS) (Atlanta Biologicals). Uridine (Sigma Chemicals) was dissolved in ethanol due to its poor solubility in water. Three different concentrations of uridine were studied: 50 μM, 100 μM and 200 μM. All the cultures were maintained at 37 °C and 6.5% CO_2 in a humidified atmosphere. The cells were subcultured at 2-day intervals.

Measurement of neurite outgrowth

The N2a cells were plated in tissue culture plates at an initial concentration of 50,000 cells per plate containing DMEM medium with 1X L-glutamine, 1X penicillin-streptomycin, 10 mM of glucose, and 10% of FBS for 48 hours. To examine the effects of uridine on neurite outgrowth, the medium was carefully replaced with FBS-free medium and either 50 μM, 100 μM or 200 μM of uridine in ethanol along with an ethanol-alone counterpart for each concentration. The cells were photographed using an Amscope MU 1400-CK digital camera attached to phase contrast microscope. Uridine's effect on neuronal growth was measured based on percent neurite-bearing cells, average neurite length and average combined length. The percentage of neurite-bearing cells is the number of neurite-bearing cells divided by the total number of cells in a field, multiplied by 100. Neurite outgrowths, defined as axon-like extensions, were

quantified using ImageJ software by tracing the axon extension and recording their lengths. Neurite length was measured in at least 100 cells per plate. Photos were taken from 12 different quadrants evenly distributed throughout the plate. Four photos in each quadrant plus two extra photos yields 50 photos per plate. Only axon extensions measuring 30 μm or more where counted for percent neurite-bearing cell. Average neurite length was calculated by measuring the longest axon extension per photo, but all measurements were used for combined neurite length.

Statistical analysis

This experiment consisted of three separate trials with different N2a cultures and reagents each time. All the experimental data were expressed as mean ± standard error. Statistical differences between groups were calculated by one-way analysis of variance (ANOVA).

Results and discussion

Uridine affects neurite outgrowth in N2a cells

Uridine has been recognized as one of the main bioactive compounds in the medicinal mushroom *Pleurotus giganteus* (GOM). We examined uridine's effects to promote neuronal outgrowth in N2a cells. The N2a neuroblastoma cell line is derived from mouse C1300 tumor and differentiated into neuron-like cells [21]. The cells contain a type of neurofilament used as a structural axon protein. Other studies have shown that neurofilament proteins were expressed in N2a cells after treatment with GOM extract [13]. We used the N2a cell line as an *in vitro* model for this study To determine the optimum uridine concertation for neurite outgrowth activity, pure uridine, at the concentrations of 50 μM, 100 μM and 200 μM, was tested. The cells were incubated for 48 hours in medium containing uridine dissolved in ethanol or ethanol alone (vehicle). The cells were then photographed and measurements were taken based on three different neural outgrowth parameters using ImageJ software.

Incubation of N2a cells with ethanol alone had no effect on the percentage of neurite-bearing cells as compared to cell growth in medium alone (Fig 1). Treatment with uridine significantly ($p<0.001$) increased neurite outgrowth for all three

concentrations (Figs 1 and 2). Concentrations of 50 μM induced 34.8% ± 13.1% growth in neurite-bearing cells and 200 μM induced 36.1± 15% growth, both showed significant differences when compared to growth in the medium alone ($p<0.001$). Uridine at a concentration of 100 μM stimulated the highest ($p<0.001$) percentage of neurite-bearing cells 39.98±13.48%, almost double the amount seen in the ethanol control (Fig 3). The increase in uridine concentration from 100 μM to 200 μM did not cause additional neurite outgrowth stimulation. It actually caused a slight decrease in the percent of neurite-bearing cells. Previous studies have shown that GOM has a uridine concentration of 1.5 μM but can increase neural growth in a dose-dependent manner, up to 100 μM [12]. These findings support our results that 100 μM is the ideal concentration of uridine for optimal neural growth. Therefore, the optimum uridine concentration of 100 μM was used for the subsequent data analyses.

The mean for the longest neurite extension of N2a cells as well as the longest neurite/axon extension per photo were also measured. Given that 50 photos were taken per plate and three trials were performed, a total of 150 neurons were measured for the effects of uridine, medium and ethanol alone. N2a cells treated with uridine significantly increased ($p<0.001$) neurite extension as compared to cells incubated with ethanol alone (Fig 4). In addition, the combined length of all axon extensions showed a significant difference ($p<0.001$) when treated with uridine (Fig 5). These results suggest uridine promotes neurite outgrowth in differentiating N2a cells with an optimum concentration of 100 μM. Former studies have shown the rate at which brain neurons form new dendritic spines depend upon three limiting compounds: uridine, DHA and choline. All three compounds are precursors of the phosphatides in neural membranes. Uridine supplements can increase brain phosphatide levels. Moreover, uridine can be an agonist for P2Y2 receptors stimulating the production of synaptic proteins [22]. Another study suggests that when uridine binds to P2Y receptors, it stimulates the MEK/ERK and PI3K/AKt/mTOR pathways [12].

Conclusion

The results from this study showed that treatment of N2a cells with uridine increased the percentage of neurite-bearing cells, neurite extension and combined length of neurites per cell. The optimum uridine concentration on neurite outgrowth was

100 μM. Future *in vivo* studies should be performed in order to examine the beneficial effects of uridine in animal models to help elucidate its therapeutic potential in neurodegenerative diseases.

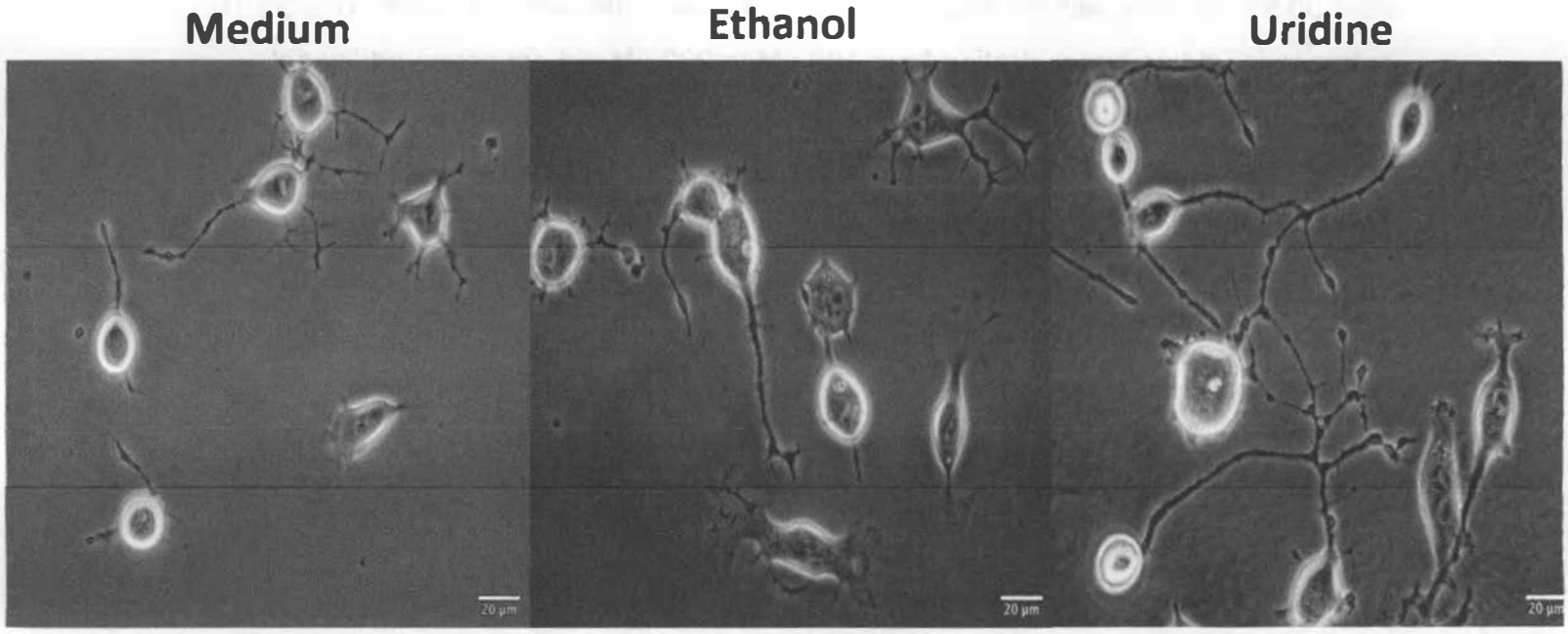

Figure 1. Phase contrast photographs of N2a neurites incubated in medium alone, medium containing ethanol (vehicle) and 100 μM of uridine.

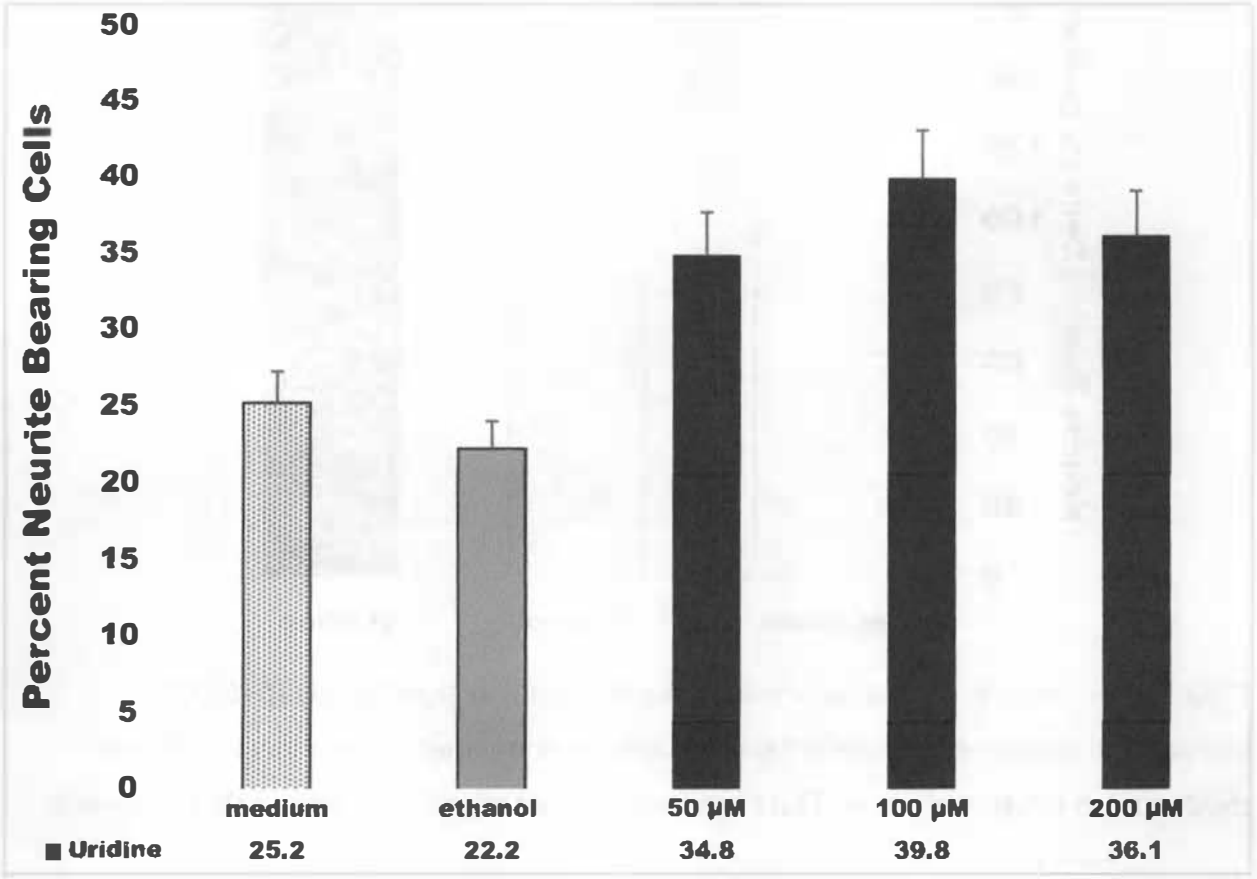

Figure 2. Incubation of N2a cells with uridine significantly ($p<0.001$) increased the percentage of neurite-bearing cells as compared to cells incubated with medium alone or ethanol. Three uridine concentrations (50 μM, 100 μM and 200 μM) are indicated in black. Uridine at a concentration of 100 μM stimulated the highest ($p<0.001$) percentage of neurite-bearing cells (39.98±13.48%) with almost double the amount of the ethanol control. Data are mean +/- standard error from 3 different trials.

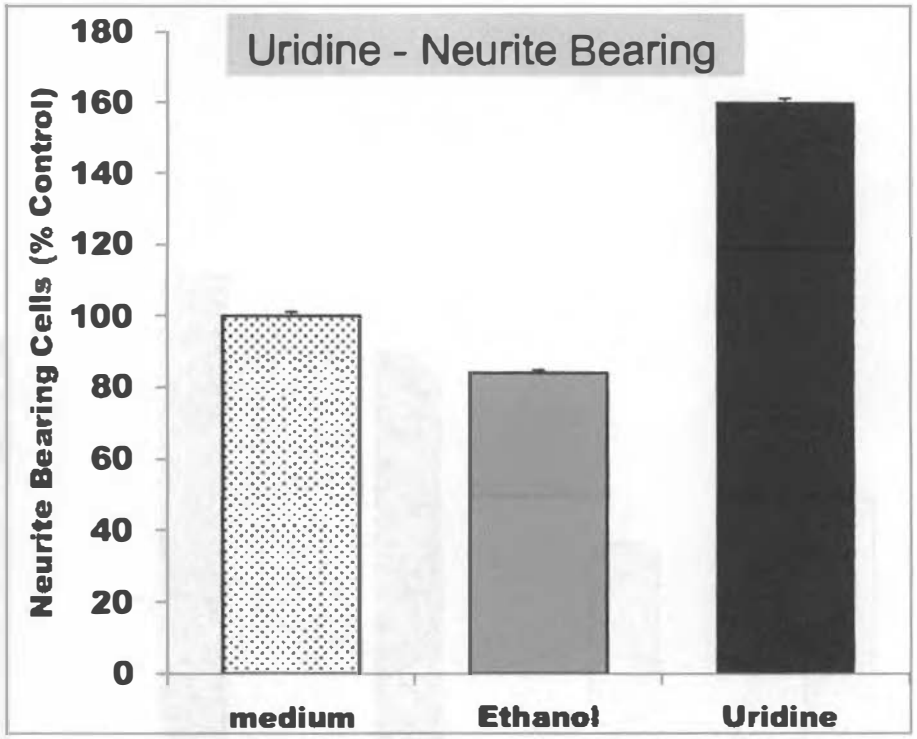

Figure 3. Incubation of N2a cells with 100 μM of uridine significantly ($p<0.001$) increased the percent of neurite bearing cells as compared to cells incubated with medium and ethanol controls. Data are mean +/- standard error from 3 different trials.

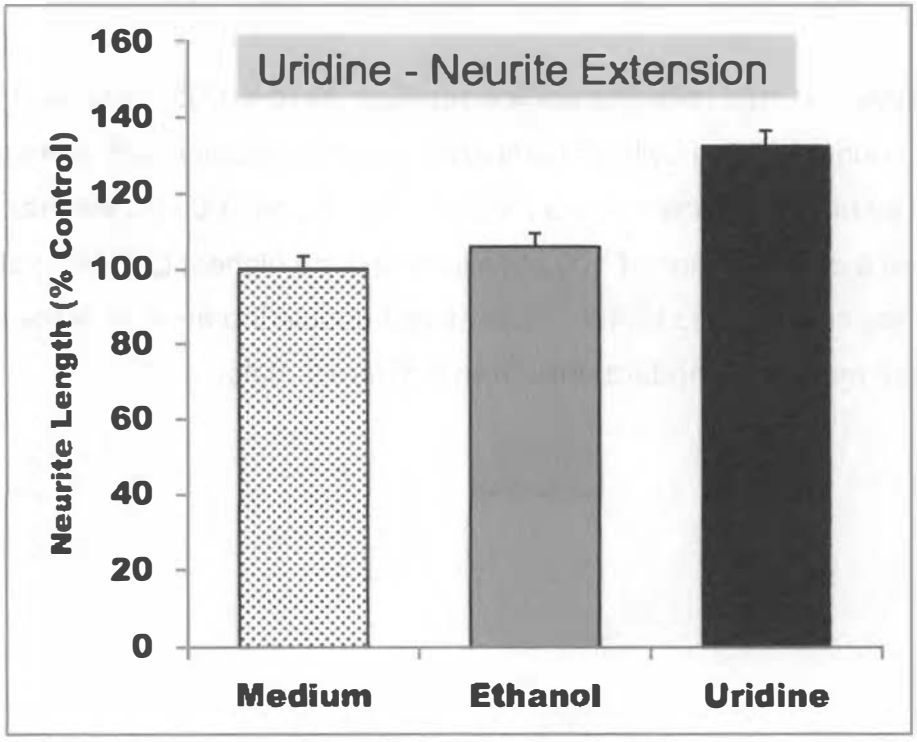

Figure 4. Incubation of N2a cells with 100 μM of uridine significantly ($p<0.001$) increased neurite length as compared to cells incubated with medium and ethanol controls. Data are mean +/- standard error from 3 different trials.

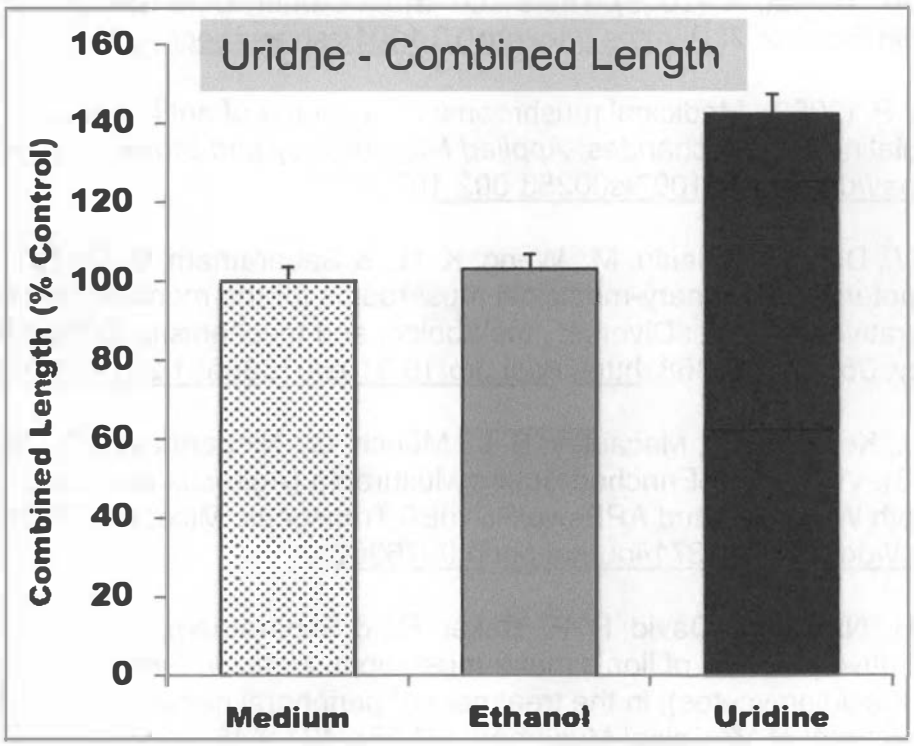

Figure 5. Incubation of N2a cells with 100 μM of uridine significantly ($p<0.001$) increased combined length of neurite-bearing cells as compared to cells incubated with medium and ethanol controls. Data are mean +/- standard error from 3 different trials.

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