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URIDINE PROMOTES NEURITE OUTGROWTH IN NEUROBLASTOMA CELLS

J. Spathies, G. C. Tucker* and B. P. Nathan

Department of Biological Sciences, Eastern Illinois University, 600 Lincoln Avenue, Charleston, IL 61920, USA
*Corresponding Author's email: gctucker@eiu.edu

ABSTRACT

Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are the main causes of age-related dementia. These diseases can be due to neuronal cell death and/or impairment of neuronal growth and connections. 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, we 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 ($p \leq 0.001$) and exhibited a significant increase in neurite-bearing cells ($p \leq 0.001$). The peak neurite promoting effect of uridine was at 100 μM. Our results suggest uridine promotes neurite outgrowth in N2a cells ($p \leq 0.001$). Future studies are required to identify the mechanism(s) behind therapeutic potential of uridine on neurodegenerative diseases.

Keywords: Uridine, giant oyster mushroom, *Pleurotus giganteus*, neurite outgrowth, herbal medicine, Neuro2a, nerve regeneration, Alzheimer's disease, Parkinson's disease.

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INTRODUCTION

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 disease (AD)and Parkinson's disease (PD), is continuing to increase at a rapid rate (Hebert *et al*., 2003). A large portion of the geriatric population will experience some form of dementia-related illnesses, increasing the economic burden of heathcare. With the central nervous system's (CNS) limited ability to repair itself after injury or progressive disease-related damage, there is currently no treatment for recovering lost CNS function. Most therapeutic medicine found to prevent neurodegenerative disease cannot cross the blood-brain barrier (Allen *et al*., 2013; Daneman *et al*., 2015). Given there is no treatment or cure for neurodegenerative diseases, like AD and PD, many people are turning to a more holistic approach.

One approach to this issue is to address age-related diseases and to act towards their prevention through dietary supplements and functional foods. An increasing amount of research has recently focused on functional foods and their bioactive constituents in our everyday diet (Khan and Tania 2012). 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 (Wasser, 2002). A community-based study in Singapore focused on men and women,60 years old and older, who consumed two portions of mushrooms per week. These individuals had a 43 % reduced risk of developing mild cognitive impairment (MCI), a brain condition that may lead to more serious diseases such as AD (Feng *et al*. 2019).

Culinary-medicinal mushrooms have been used for their bioactive, secondary metabolites to reduce amyloid-induced neurotoxicity, neurite outgrowth stimulation, nerve growth factor synthesis, neuroprotective function, antioxidant, and anti-inflammatory effects (Phan. *et al*. 2015). Vitamin D-enriched white button mushrooms, *Agaricus bisporus* (J.E.Lange) Imbach, have been shown to improve the memory in a mouse model for AD (Bennett *et al*. 2013). Mushrooms have even been found to produce compounds that promote neurite outgrowth. As an example, erinacines and hericenones, isolated from the mushroom *Hericium erinaceus* Persoon, commonly called lion's mane, for their neuroprotective properties (Wong *et al*. 2012). Since these brain-improving compounds can only be isolated from the mushrooms that produce them, an increasing amount of research is needed to identify the mechanism behind their medicinal properties.

Mushrooms of the genus *Pleurotus* are widely consumed worldwide, for their flavor and high nutritional value (Khan and Tania 2012). *Pleurotus giganteus* (Berk.) Karunarathna and Hyde, 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 (Lee *et al*., 2009). Many *in vitro* studies of GOM have evaluated its anticancer, antioxidative, antifungal, hepatoprotective, and neurite outgrowth capabilities (Karunarathna *et al*. 2012; Phan *et al*. 2013, 2015; Baskaran *et al*. 2017). The GOM extracts exhibited neurite outgrowth in rat pheochromocytoma (PC12) and N2a cells (Phan *et al*. 2012, 2013). The main bioactive constituent of GOM is believed to be uridine.

Uridine is an RNA nucleotide and has been identified in several mushroom species (Yang *et al*., 2012). Specifically, it was recognized as one of the main bioactive compounds in the medicinal mushroom species, *Cordyceps militaris* (L.) Link, containing 45.4 mg/kg extract of uridine (Das *et al*., 2010). Uridine is also present in breast milk (Thorell *et al*., 1996) and, as a nucleotide; it is reported to have important physiological roles in breastfeeding infants (Leach *et al*., 1995). Uridine contributes to brain phosphatidylcholine synthesis via the Kennedy pathway (Cansev, 2016; Pooler *et al*., 2005). Its uptake into the brain and through the blood-brain barrier 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 (Phan *et al*., 2015).

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

MATERIALS AND METHODS

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 incubator. The cells were subcultured at 2-day intervals (Nathan and Tucker 2022).

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 replaced with FBS-free medium containing 1X L-glutamine, 1X penicillin-streptomycin, 10 mM of glucose and either 50 μM, 100 μM or 200 μM of uridine in ethanol along with an ethanol-alone counterpart for each concentration. The uridine concentration tested in our study was based on previously reported uridine concentration of 1.6-1.8% (g/100g) in Giant Oyster Mushroom extract, which corresponds to 1.5 mM of uridine (Phan *et al*., 2015). This study also showed that 100 mM elicited the maximum neurite outgrowth in NGF-treated neuronal cultures. The cells were photographed using an Amscope MU 1400-CK digital camera attached to a phase-contrast microscope. Our methods follow those used in a previous study of plant derived compounds (Asiatic and Madecassic acids) on cultured neurite growth (Nathan and Tucker 2019).

We measured three parameters of neuronal growth: percent neurite-bearing cells, longest neurite length, and average combined length (Nathan and Tucker 2022). Neurite length was measured in at least 50 cells using ImageJ © software. The percentage of neurite-bearing cells is the number of neurons with a neurite extension measuring at least 30 μm divided by the total number of cells in a field and then multiplied by 100%. For measuring the longest neurite and combined length of neurites, photos were taken from 12 different quadrants evenly distributed throughout the plate. Given that 50 photos were taken per plate and three trials were performed, a total of 150 neurons were measured for each treatment condition (Nathan and Tucker 2022).

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

RESULTS AND DISCUSSION

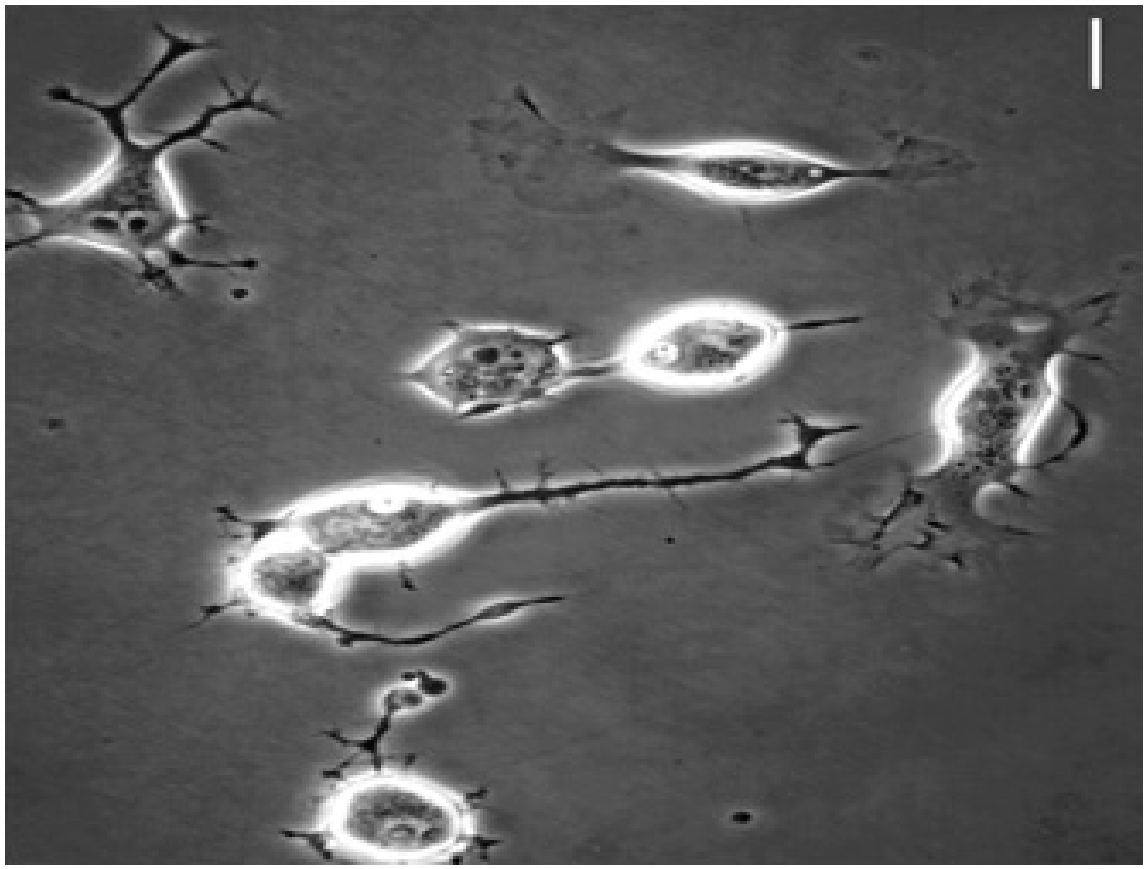
Uridine has been recognized as one of the main bioactive compounds in the medicinal mushroom *Pleurotus giganteus* (GOM). We examined uridine's effects to promote neuronal outgrowth in N2a cells. The N2a neuroblastoma cell line is derived from mouse C1300 tumor and differentiated into neuron-like cells (Radio *et al*., 2008). The cells contain an intermediate filament, neurofilament 200, used as a structural axonal protein (Posmantur *et al*., 2009). Other studies have shown that neurofilament 200 were expressed in N2a

cells after treatment with GOM extract (Phan *et al*., 2013). We used the N2a cell line as an *in vitro* model for this study to determine the effect of uridine on neurite outgrowth.

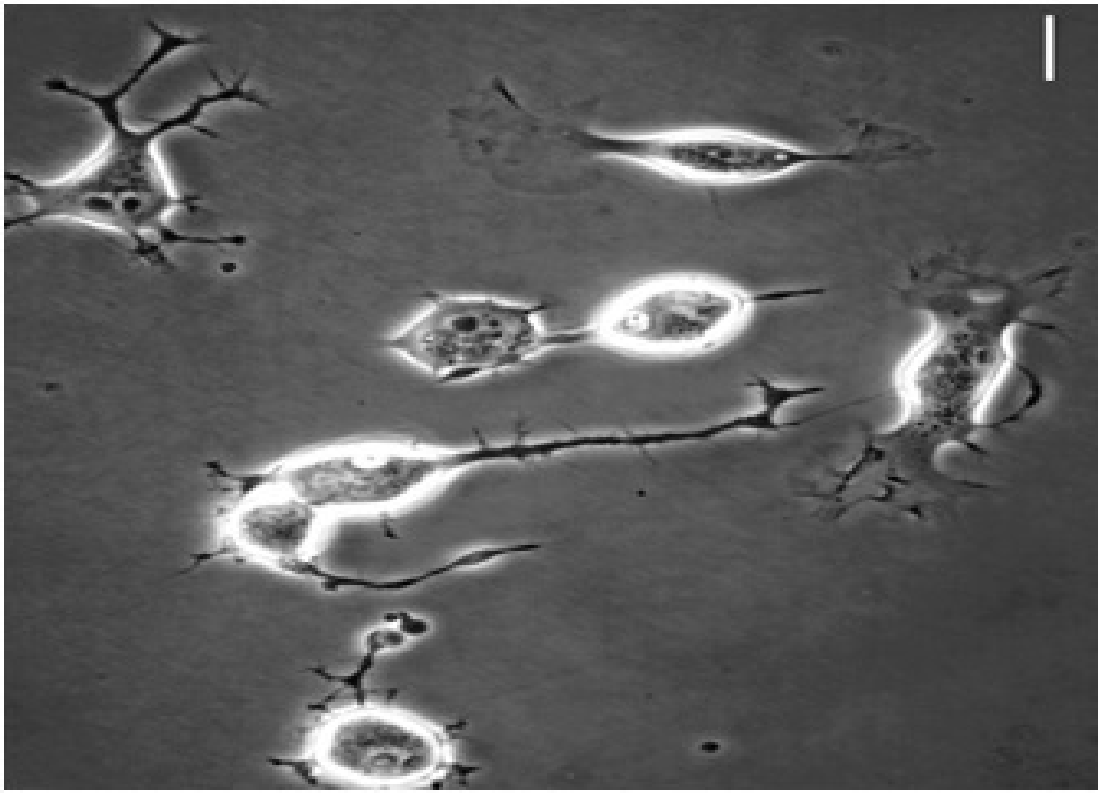
Incubation of N2a cells with ethanol alone (vehicle) had no effect on the percentage of neurite-bearing cells as compared to cell grown in medium alone (Figure 1). Treatment of N2a cells with uridine significantly ($p \leq 0.001$) increased the percentage of neurite bearing cells as compared to cells grown in vehicle alone (figure 2A). Uridine at a concentration of 100 μM stimulated the highest percentage of neurite-bearing cells, almost double the amount seen in the vehicle control. The increase in uridine concentration from 100 μM to 200 μM caused a slight decrease in the percent of neurite-bearing cells. Previous studies have shown GOM has a uridine concentration of 1.5 μM, and can increase neural growth in a dose-dependent manner, up to 100 μM (Baskaran *et al*. 2017). These findings support our results that 100 μM is the ideal concentration of uridine for optimal neural growth.

N2a cells treated with uridine significantly increased ($p \leq 0.001$) neurite length as compared to cells incubated with vehicle alone (Figure2B). In addition, the combined length of all neurite extensions showed a significant increase ($p \leq 0.001$) when treated with uridine as compared to the vehicle alone (Figure2C). These results suggest uridine promotes neurite outgrowth in N2a cells. Previous 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 (Wurtman *et al*., 2010). Another study suggests when uridine binds to P2Y receptors, it stimulates the MEK/ERK and PI3K/AKt/mTOR pathways, which in turn increases neuronal growth (Phan *et al*., 2015).

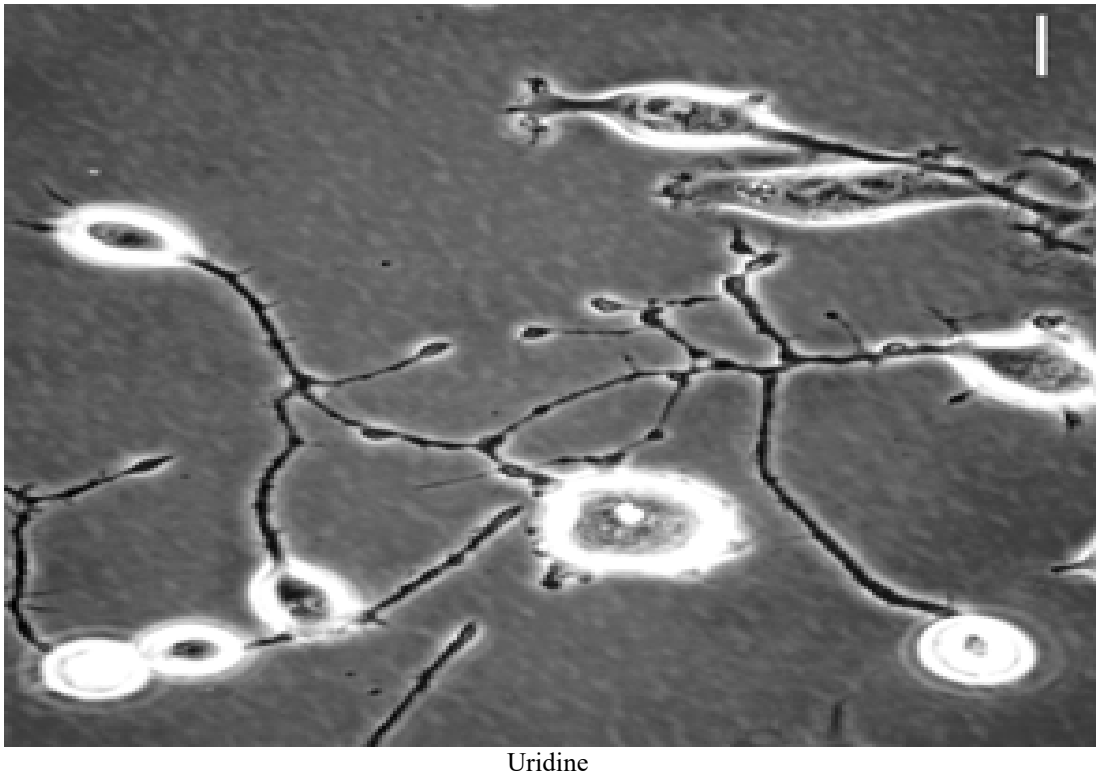
The results from our study demonstrate that treatment of N2a cells with uridine increased the percentage of neurite-bearing cells, neurite extension, and combined length of neurites per cell. The optimum uridine concentration on neurite outgrowth was 100 μM. Future *in vivo* studies are desirable, to examine the beneficial effects of uridine in animal models and further elucidate its therapeutic potential in neurodegenerative diseases.



Medium 1



Ethanol



Uridine

Figure 1–Representative phase contrast micrographs of N2a cells incubated in medium alone (A), medium containing ethanol (vehicle, B) and 100 μM of uridine (C). Scale bar = 20 µm.

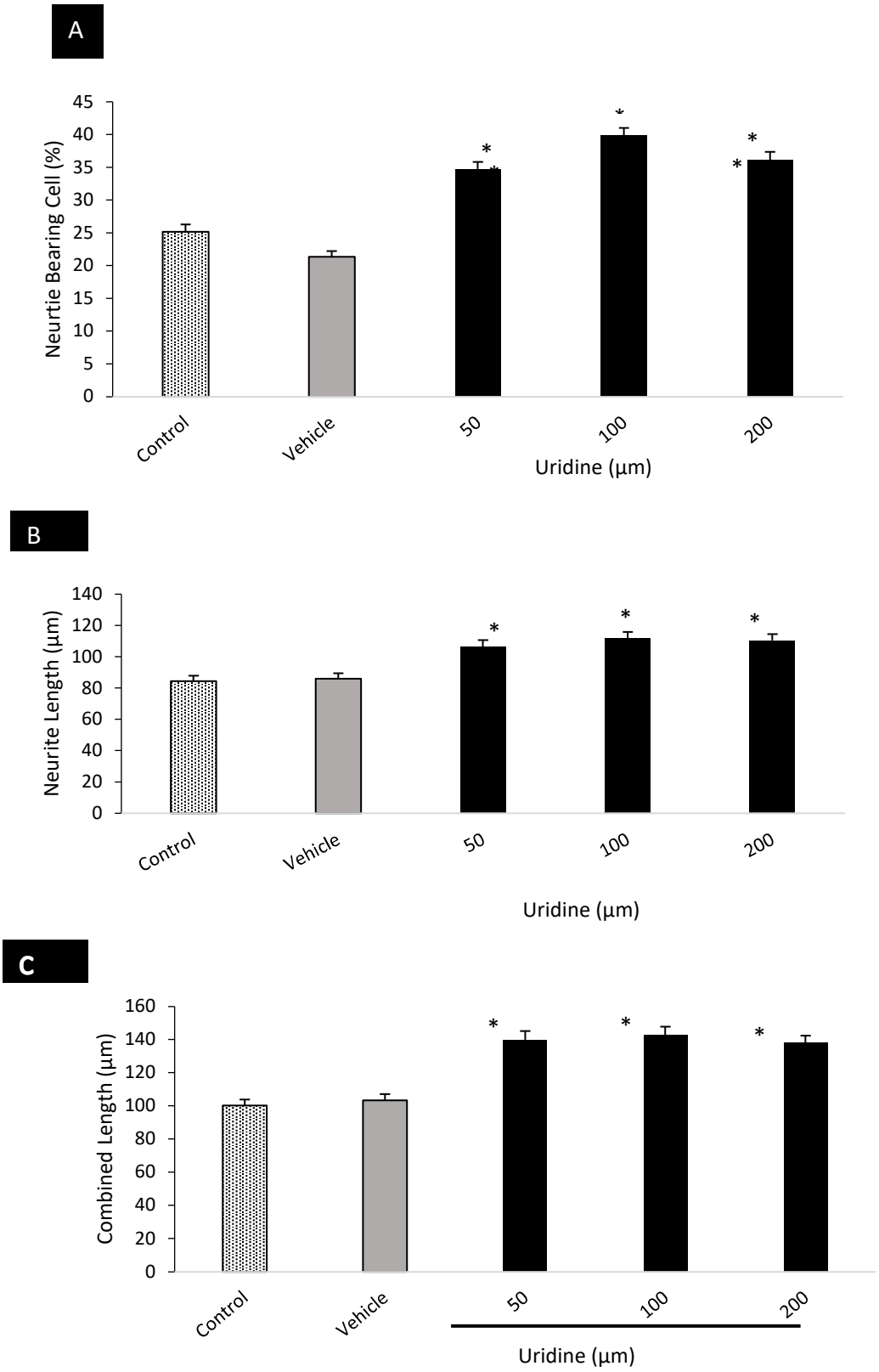


Figure 2 - Incubation of N2a cells with uridine significantly (*p≤0.001) increased the percentage of neurite bearing cells (A), neurite length (B), and combined length of neurite (C) as compared to cells incubated with ethanol alone (vehicle). Data are mean +/- SE from three different experiments.

List of abbreviations: GOM, *Pleurotus giganteus*; N2a, Neuro2a murine neuroblastoma; CNS, Central nervous system; and MCI, mild cognitive impairment.

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