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1,3-Dinitrobenzene neurotoxicity – Passage effect in immortalized astrocytes

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ABSTRACT

Age-related disturbances in astrocytic mitochondrial function are linked to loss of neuroprotection and decrements in neurological function. The immortalized rat neocortical astrocyte-derived cell line, DI-TNC$_1$, provides a convenient model for the examination of cellular aging processes that are difficult to study in primary cell isolates from aged brain. Successive passages in culture may serve as a surrogate of aging in which time-dependent adaptation to culture conditions may result in altered responses to xenobiotic challenge. To investigate the hypothesis that astrocytic mitochondrial homeostatic function is decreased with time in culture, low passage DI-TNC$_1$ astrocytes (LP; #2–8) and high passage DI-TNC$_1$ astrocytes (HP; #17–28) were exposed to the mitochondrial neurotoxicant 1,3-dinitrobenzene (DNB). Cells were exposed in either monoculture or in co-culture with primary cortical neurons. Astrocyte mitochondrial membrane potential, morphology, ATP production and proliferation were monitored in monoculture, and the ability of DI-TNC$_1$ cells to buffer K^+-induced neuronal depolarization was examined in co-cultures. In HP DI-TNC$_1$ cells, DNB exposure decreased proliferation, reduced mitochondrial membrane potential and significantly decreased mitochondrial form factor. Low passage DI-TNC$_1$ cells effectively attenuated K^+-induced neuronal depolarization in the presence of DNB whereas HP counterparts were unable to buffer K^+ in DNB challenge. Following DNB challenge, LP DI-TNC$_1$ cells exhibited greater viability in co-culture than HP. The data provide compelling evidence that there is an abrupt phenotypic change in DI-TNC$_1$ cells between passage #9–16 that significantly diminishes the ability of DI-TNC$_1$ cells to compensate for neurotoxic challenge and provide neuroprotective spatial buffering. Whether or not these functional changes have an *in vivo* analog in aging brain remains to be determined.

1. Introduction

Notable age-related declines in mammalian mitochondrial responses to the cellular environment have been linked to the normal and accelerated degeneration of brain and other organs. Aging itself introduces cumulative declines in function in brain mitochondria, including acquired mitochondrial DNA mutations (Kennedy et al., 2013), decreased mitochondrial membrane potential (Lafrance et al., 2005), and elevated protein oxidation (Perluigi et al., 2010), among others. However, accelerated neural degeneration can result from chronic abnormal metabolic disturbances such as diabetes (Moreira et al., 2003): ultimately increasing the rate of normal mitochondrial aging. The brain not only has to compensate for its own age-related mitochondrial dysfunction, but also those imposed over time by systemic metabolic insults. Because neurons are postmitotic and depend on the ability of the macroglia to maintain viability through the active management of the extracellular environment, astrocytes play a major role long-term compensatory neuroprotection.

Brain mitochondrial metabolism decreases in both neurons and glia of aging humans (Boumezbeur et al., 2010) and is likely responsible for the steady overall loss of function due to the

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dependence of neurons on astrocytic metabolic and physical support. Astrocytes participate actively in a variety of neuroprotective functions that include scavenging excess extracellular glutamate (Rosenberg and Aizenman, 1989), and regulating extracellular calcium and potassium concentrations (Queiroz et al., 1997). Astrocytic function relies on the presence of mitochondria that have an intact membrane potential for the formation and transport of ATP: which is, in turn, required for the maintenance of neuroprotective functions (Volobouéva et al., 2007). Thus, the accumulation of (bio)chemical and physical insults to astrocytic mitochondria over time can result in a deterioration in ATP-requiring neuroprotective astrocyte functions in older mammals.

1,3-Dinitrobenzene (DNB) is an industrial synthetic intermediate that produces focal, bilaterally symmetrical spongiform lesions in the brainstem similar to those seen in acute energy deprivation syndromes (Philbert et al., 1987). It has been established that astrocytes within the brainstem are a principal cellular target in DNB exposure (Philbert et al., 1987), and that astrocyte mitochondria are a sensitive organellar target in DNB exposure (Steiner and Philbert, 2011; Miller et al., 2011; Phelka et al., 2003, 2006; Tjalkens et al., 2000). Astrocyte responses to DNB include swelling of foot processes (Philbert et al., 1987), induction of oxidative stress (Romero et al., 1995), and increased consumption of glucose while simultaneously increasing the production of lactate (Romero et al., 1996). While these astrocyte-specific responses have been identified, the targeting of these cells by DNB is incompletely understood. It is unlikely that a singular mechanism of DNB-induced mitochondrial toxicity exists in astrocytes, as evidence of DNB-induced damage to mitochondria ranges from inhibition of pyruvate dehydrogenase (Miller et al., 2011) to oxidative modifications to the mitochondrial proteome (Steiner and Philbert, 2011). Astrocytes release the nucleoside adenosine as a neuroprotective mechanism (Lian and Stringer, 2004); extracellular adenosine increases significantly in immortalized astrocyte monoculture with corresponding increases in DNB concentrations (Wang et al., 2012), suggesting that DNB exposure causes astrocytes to expend their energetic resources toward neuroprotective mechanisms, perhaps at the expense of other cellular roles. Exposure to neurotoxicants that selectively target astrocyte mitochondria, such as DNB, may therefore exacerbate existing or acquired metabolic imbalances as a result of aging.

This study examines the role of time in culture (a potential surrogate for aging *in vivo*) as a signficant factor in diminishing the capacity of astrocytes to maintain local homeostasis and, hence neuroprotection. Additionally, these studies provide evidence of specific changes to mitochondrial dynamics and morphology related to immortalized astrocyte passage number that may be sentinels of compromised function.

2. Materials and methods

2.1. Cell culture

Immortalized astrocytes (DI-TNC$_1$) were obtained from the American Type Culture Collection and maintained in a humidified incubator at 37 °C and 5% CO_2. Growing media for DI-TNC$_1$ astrocytes consisted of Dulbecco's Modified Eagle's Medium (DMEM, 25 mM D-Glucose, 1 mM sodium pyruvate), 10% Fetal Bovine Serum, and 1% Antibiotic/Antimycotic (10,000 units/mL penicillin, 10,000 μg/mL streptomycin, 25 μg/mL Fungizone® Antimycotic (Gibco)). Immortalized astrocytes were plated in 25 cm^2 flasks at a density of 2.1×10^5 cells. Approximately 48 h later, the cells had reached confluency, and were then used for experiments or subcultured to another passage (each subculture event = 1 passage). Media and additives were vacuum filtered for sterilization prior to use. Primary neurons were isolated from E18 Sprague-Dawley rat cortex, obtained from BrainBits, LLC and maintained in a humidified incubator at 37 °C and 5% CO_2. Isolation of neurons was performed according to the protocol by BrainBits. Briefly, glass coverslips were coated with 50 μg/mL Poly-D-Lysine and allowed to adhere at least 1 h at 37 °C. Coverslips were then subsequently rinsed with sterile water 3 times and then allowed to dry under the sterile hood. Cortices were dissociated with 2 mg/mL papain in Hibernate E media without calcium (BrainBits, LLC) using a fire-polished glass Pasteur pipette. Papain solution was removed and Hibernate E media (BrainBits, LLC) was added to the dissociated tissue. Tissue was allowed to settle, supernatant was spun down to pellet the neurons, and then the pellet was resuspended in Neurobasal media supplemented with B-27, Glutamax, and 1% Antibiotic/Antimycotic (all from Invitrogen). Neurons were diluted and then plated at 1.2×10^5 cells per coverslip. After 3 h, half of the media was removed and fresh supplemented Neurobasal media was used to rinse the coverslips 3 times. Media was then changed every 4–5 days afterward.

2.2. Mitochondrial flickering detection and analysis

DI-TNC$_1$ astrocytes were plated onto glass coverslips and grown to ~30–50% confluence (18–24 h) in an incubator. Growing media was replaced with DNB dosing media (serum-free DMEM). One hour after exposure to DNB, 10% FBS was supplemented back into the media. Fifteen minutes prior to imaging, the dosing media was aspirated, cells were rinsed gently with Dulbecco's phosphate buffered saline (DPBS), and 100 nM tetramethylrhodamine methyl ester (TMRM) (in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/Hanks' Balanced Salt Solution: HEPES/HBSS buffer) was added. After a 15-min incubation period, the TMRM solution was replaced with HEPES/HBSS buffer and the cells were imaged. Cells were imaged on an Olympus IX-800 disk scanning unit (DSU) microscope at intervals of 500 ms for 60 s. Light intensity of the mercury lamp was maintained at a constant wattage output, measured by a light meter, so as to maintain imaging consistency and to control for phototoxicity. During imaging, the cells were maintained in an environmental chamber (Pathology Devices), set at 5% CO_2, 37 °C, and 60% humidity. The microscope was controlled using SlideBook software (version 5.0), and image loops were reviewed immediately following image acquisition.

2.3. Morphological analysis

Microscopic images were converted to binary images using MATLAB (R2010a). Mitochondrial morphology was quantified by calculating the mitochondrial form factor, *FF*, using which is defined as:

$$FF = \frac{\pi}{4}\frac{CL}{W}$$

where *CL* is centerline and *W* is average width. The centerline of a mitochondrion was extracted from the binary image using morphological thinning operation. The average width of a mitochondrion was then calculated as area divided by centerline length. Centerline to average width ratio was multiplied by $\pi/4$ to allow the form factor of a circle to be 1.

2.4. Bromodeoxyuridine (BrdU) proliferation assay in DI-TNC$_1$ cells

DI-TNC$_1$ astrocytes were plated onto 96-well plates at 5×10^3 cells per well and grown to confluence (24 h) in an incubator. Growing media was replaced with DNB dosing media (serum-free DMEM). One hour after exposure to DNB, 10% FBS was supplemented back into the media. The manufacturer's instructions for the BrdU ELISA (Roche) were followed. Briefly, BrdU