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Particulate Matter Neurotoxicity in Culture is Size-Dependent

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Introduction

Particulate matter (PM) has been epidemiologically associated with death and cardiopulmonary illnesses in the elderly and in those individuals with pre-existing respiratory conditions (e.g., smokers, asthmatics, etc.) (Brook *et al.* 2010; Krewski *et al.* 2009; Pope and Dockery 2006). Numerous animal studies indicate that inhalation of PM results in inflammatory toxicity in the airways and cardiovascular effects (Chen *et al.* 2010; deHaar *et al.* 2006; Maier *et al.* 2008; Oberdorster *et al.* 2000). Although the historical focus of PM toxicity has been on these cardiopulmonary targets, it is now appreciated that inhaled nano-size particulates can quickly exit the lungs and enter the circulation where they distribute (i.e., liver, kidneys, testes, lymph nodes, brain) (Kreyling *et al.* 2004; Oberdorster *et al.* 2002a; Oberdorster *et al.* 2002b) and these organ systems through oxidative stress (Pohjola *et al.* 2003; Samet *et al.* 2004; Schulz 2006).

Because of its high energy demands and its inherently low level of endogenous scavengers (e.g., vitamin C, catalase, superoxide dismutase etc.) the brain is extremely sensitive to free radical damage (Halliwell 1992; Tanaka 1997). In the central nervous system (CNS), oxidative stress is largely mediated by microglia, which are macrophage-like, phagocytic cells that are activated by a broad range of stimuli, including PM (Block *et al.* 2004; Sama *et al.* 2007) and nanoparticles (Long *et al.* 2006). Once activated, the microglia produce multiple reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and

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peroxy-nitrites (Block *et al.* 2007; Fariss *et al.* 2005) that can diffuse from their plasma membrane and damage nearby neurons. Dopaminergic neurons are especially vulnerable to oxidative stress damage since they support a high synaptic network within the mesencephalic brain (striatum) and because dopamine metabolism in itself generates ROS (Double *et al.* 2010; Gao *et al.* 2002; Gonzalez-Hernandez *et al.* 2010; Jackson-Lewis and Smeyne 2005; Mitchell *et al.* 1999). The striatum is a brain area, which houses large populations of dopaminergic neurons and a disproportionately large population of microglia (Hirsch 1994; Koutsilieri *et al.* 2002).

The major components of PM include transition metals, sulfate and nitrate ions, organics, minerals, adsorbed gases, and biocontaminants (e.g., endotoxins, mold, pollen etc.), attached to a core of carbonaceous material. The size of the particulates found in PM has been inversely related to its inflammatory damage (deHaar *et al.* 2006; Oberdorster 1996; Pedata *et al.* 2010; Valavanidis *et al.* 2008), with smaller, ultrafine size particles being more toxic to the target tissue. Mechanisms to explain this phenomenon may be physiological and/or physicochemical in nature. The current study examines if this phenomenon (size-dependent toxicity) can be reproduced in isolated dopaminergic neurons (N27) and cultures dissociated from the embryonic rat brain striatum sized-PM material.

Materials and Methods

Collection of size-fractionated ambient PM Samples

The PM samples were collected over a two week period in August 2005, within the Sterling Forest State Park (Tuxedo, NY). The park is a largely undeveloped woodland park located approximately 50 km north-west of Manhattan. It is bordered by a lightly traveled two-lane road and there are no large power generators or industrial operations within 20 miles of the collection site. The ambient air in Sterling Forest is considered as regional background PM and is representative of the ambient air over the Virginia-Maine megalopolis (Maciejczyk and Chen 2005). The air samples were collected using the Multi-Orifice Uniform Deposit Impactor (MOUDI) (MSP, St. Paul, MN) which size-classifies the PM material as it is collected. Sized-samples were classified as Accumulation Mode Fine (AMF) ($>0.18\text{--}1\ \mu\text{m}$) or Ultrafine (UF) ($<0.18\ \mu\text{m}$). The ambient air in Sterling Forest is considered as regional background PM and is representative of the ambient air over the Virginia-Maine megalopolis. The volume-size distribution of ambient air collected in this area of the park during the spring and summer is bimodal with mean diameters in both the AMF and UF range. The volume-size distribution of ambient air collected from Sterling Forest are described in detail in (Maciejczyk *et al.* 2005).

Samples were collected on pre-weighed Teflon filters and then immersed in Dubelco's Minimal Essential Media to yield a concentration of 500 $\mu\text{g}/\text{ml}$. Aliquots of these suspensions were diluted to stock concentrations for *in vitro* exposure. Care was taken not to sonicate or vigorously vortex the suspensions to preserve their original size at the time of collection.

Analyses for 35 elements were performed New York University's A. J. Lanza Laboratory by non-destructive XRF (ModelEX-6600-AF; Jordan Valley) using five secondary fluorescers (Si, Ti, Fe, Ge, and Mo), and spectral software XRF2000v3.1 (US EPA and ManTech Environmental Technology). The major components in the test samples consisted of sulfur (most likely as secondary aerosol particles) and elements associated with soil, (e.g., Si, Al, Ca, and Fe) (data not shown). A detailed composition analysis of the ambient samples collected at Sterling Forest has been reported previously (Maciejczyk and Chen 2005).

Cells and Culture Maintenance

The PM samples were tested on N27 neurons, an immortalized dopaminergic immortalized neuron from the mesencephalic brain of wild type, Sprague-Dawley rats (Zhou *et al.* 2000). N27 neurons were grown in RPMI 1640 media, supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (ATCC, Manassas, VA). The N27 neurons were plated in 96 well plates (Costar, Corning, Inc., Corning, NY) and grown to ~85% confluency before exposure to the individual particulate samples. All cell culture reagents were purchased from InVitrogen, Carlsbad, CA.

Primary cultures of Sprague Dawley embryonic striatum were purchased from BrainBits™ (<http://www.brainbitsllc.com>). Upon receipt the tissues were triturated in Neurobasal™, a serum-free media supplemented with glutamine, plated on poly-lysine-coated BTM 96 well plates (Nagle Nunc International-NUNC™, Rochester, NY), and grown to 85% confluency before exposure.

Neuronal Assays

To examine if PM was neurotoxic to isolated neurons, N27 cells were exposed to non-sonicated aliquots of AMF and UF (12.5, 25, and 50 µg/ml) for 24 hr. After exposure, cells were washed multiple times to remove extraneous particles. Neuronal loss was measured with Hoechst 33258, a nuclear stain that binds to the adenine-thymine rich regions of double stranded nuclear DNA. After exposure, cells were washed with Hank's basic salt solution, and then lysed by alternating temperatures (1 hr at 37° C followed by 1 hr -80°C). Hoechst reagent (2.0 µg/ml) was added to each well for 30 min and the plates spectrophotometrically read at 346/460nm wavelengths.

Reactive nitrogen species and nitric oxide are important physiological messengers in neuronal and cardiovascular tissues (Halliwell 1992; Mohanakumar *et al.* 2002). Inducible nitric oxide is generated from microglia (Chandra *et al.*, 2000; Fariss *et al.*, 2005) and can be measured with nitrite, a primary and stable breakdown product of nitrous oxide (Hengartner, 2000). Nitrite was measured in complex brain (striatum) cultures using Griess reagent under acidic (pH 4.0) conditions (Miranda *et al.* 2001). Plates were read using chemiluminescence.

For all spectrophotometric measurements, cells were plated in either black (fluorescence) or white (chemiluminescence) 96-well culture plates (Corning Inc., Corning, NY) to minimize light scatter. Spectrophotometers (Molecular Device, Sunnyvale, CA) included the Spectramax EM (fluorescence) and Lmax II 384 (chemiluminescence) plate readers.

Immunohistochemistry

Neurons in the striatum cultures were selectively stained with (mouse, rat, human) neuron specific enolase (NSE) (Dako Inc., Glostrup, Denmark, www.dako.com) at a 1:200 concentration according to protocol. For visualization, cells were biotinylated, linked to Streptavidin and counter-stained with a chromagen substrate according to directions given in the LSAB 2 System-HRP (Dako Inc). Support reagents were purchased from Sigma (St. Louis, MO).

Morphometry

Semi-confluent cultures (n=3 wells) of embryonic brain striatum were exposed to AMF (80 µg/ml) or UF (8 µg/ml) for 24 hr. After exposure, cultures were fixed in 3.7% formalin, immunocytochemically stained, and analyzed morphometrically for neuronal loss. For this, six photographs were taken of each well (10× apocromatic lens) using a Nikon TE300 inverted microscope fitted with a cooled-frame CCD camera (Orca I, Hamamatsu, Inc.). Each digitized image was analyzed using Metamorph 7.0 software (Molecular Device,

Sunnyvale, CA). Threshold levels of pixel stain were used to quantitate the Total Area (i.e., neuronal cell bodies and axonal plexus) of NSE staining and the values binned according to area. Shape and size parameters were also defined to describe the neuronal cell body alone and applied to the Total Area data set using the Integrated Morphometric Analysis mode. Values for the Total Area and those measuring the neuronal cell bodies were collected in AMF (80 $\mu\text{g/ml}$ and UF (8 $\mu\text{g/ml}$) or) treated cultures.

Statistical Analysis

All data were collected using SoftMax Pro 4.8 software (Molecular Devices, Sunnyvale, CA). Graphing was performed with GraphPad Prism 4.02. Data were analyzed using a one-way ANOVA with Dunnett's post-testing to determine the lowest statistically significant treatment relative to media control. Statistically significant difference is indicated (#) on the graph ($p < 0.05$) and described in the figure legends.

Results

Direct neurotoxicity

To determine if PM was neurotoxic in the absence of other cell types, dopaminergic neurons (N27) were exposed to AMF or UF (12.5, 25, 50 $\mu\text{g/ml}$) samples for 24 hr. Significant neuronal loss (as measured by Hoechst nuclear stain) occurred in response to all tested concentrations of UF ($> 12.5 \mu\text{g/ml}$) but only in response to the highest concentration of AMF (50 $\mu\text{g/ml}$), suggesting a size-dependent neurotoxicity in isolated neurons (Figure 1).

Microglia-mediated neurotoxicity

To examine if this relationship was altered in the presence of other brain cell types (e.g., microglia), primary cultures of embryonic rat striatum were exposed to either AMF (80 $\mu\text{g/ml}$) or UF (8.0 $\mu\text{g/ml}$). Equivalent increases of nitrite were measured by chemiluminescence after 24 hr exposure (Figure 2A). Microscopic examination indicated that the larger neuronal cell bodies in both the AMF and UF - treated neurons showed an apoptotic morphology (i.e., swollen cell body, fragmented nuclear material, centrally located nuclei, blebbing etc.) (Chandra et al., 2000; Fariss et al., 2005; Hengartner, 2000) (Figure 2B-D). A histogram of Total Area (neurons and axons) indicated that NSE-staining was most reduced in the larger size bins 110-130 (Figure 3A). When size and shape parameters specific for the larger neurons were applied to the analysis, a significant reduction of NSE staining occurred only in cultures treated with UF, relative to controls (Figure 3B).

Discussion

The possibility that the brain might also be targeted by PM was first raised in an editorial (Oberdorster and Utell 2002). This was followed by reports showing that nanosize particles could cross the blood brain barrier (Lockman *et al.* 2004) and physically enter the central nervous system of animals in small numbers (Kreyling *et al.* 2004; Oberdorster *et al.* 2002a; Oberdorster *et al.* 2002b; Oberdorster *et al.* 2004; Takenaka *et al.* 2001). Histological evidence of PM-related neurodegeneration was first described in the olfactory and respiratory nasal mucosa, olfactory bulb, and cortex of feral dogs living in Mexico City (Calderon-Garciduenas *et al.* 2003). In these tissues, alterations of the blood brain barrier, degenerated cortical neurons, apoptotic myelin producing cells (i.e., oligodendrocytes), and neurofibrillary tangles were noted. Immunohistochemical expressions of oxidative stress-mediated damage were found, including increased staining of NF- κB and inducible nitric oxide. Unfortunately, since the highly polluted Mexico City ambient air contained numerous atmospheric contaminants, the authors could only suggest a linkage of the pathology to the unspecified air pollutants.

Shortly after the above reports were published, experiments were designed by our laboratory to determine if animals exposed to PM under experimental conditions would show neuropathological damage. The Apo E^{-/-} “knock-out” mouse strain and its normal C57/Blk6 background strain were exposed by inhalation to concentrated ambient particulate matter (CAPs) collected from the Sterling Forest State Park (Tuxedo, NY). After 6 month exposure, the brains of the oxidative stress-prone Apo E^{-/-} were serially section and immunocytochemically stained for dopaminergic neurons. Morphometric analysis of neurons in the striatum indicated that neither air (control) nor CAPs-exposed C57/Blk6 normal mice showed significant reduction of stain. However, in the oxidative stress-prone Apo E^{-/-} mice, CAPs exposure caused a 29% reduction in the immunocytochemically stained dopaminergic neurons of the striatum relative to the air exposed Apo E^{-/-} mice (Veronesi *et al.* 2005).

Because of its critical role in oxidative stress-mediated neurodegeneration (Beal 2003; Fariss *et al.* 2005), immortalized microglial cells (BV2) from C57/Blk6 mice, were exposed to the same CAPs and their cellular and genomic response examined. The CAPs were first analyzed by XRP and ranked as high potency (HP-CAPs) or low potency (LP-CAPs) samples, depending on the presence of nickel and vanadium metals. Both samples reduced endogenous scavengers and both stimulated the release of pro-inflammatory cytokines TNF α and IL-6. However, the genomic response differed in magnitude and scope. Exposure of BV2 microglia to the nickel and vanadium enriched HP-CAPs activated numerous pro-apoptotic and inflammatory pathways such as Notch activating pathway for NF-kB signaling and up-regulated innate immune pathways involving the Toll-Like Receptors 2-8. In contrast, LP-CAPs stimulated pathways related to cellular maintenance and cellular division (Sama *et al.* 2007). These *in vitro* studies, coupled with the demonstration of neurodegeneration in the substantia nigra of Apo E^{-/-} mice unequivocally defined the brain as a target of inhaled CAPs in oxidative stress-compromised mouse model, and helped to identify the vulnerable neuronal and microglia cellular targets. Numerous clinical and experimental studies have since identified the brain to be neurochemically and neuropathologically affected by various types and sizes of PM air pollution (Block *et al.* 2004; Calderon-Garciduenas *et al.* 2007; Calderon-Garciduenas *et al.* 2008; Campbell *et al.* 2005; Campbell *et al.* 2009; Gerlofs-Nijland *et al.* 2010; Sirivelu *et al.* 2006; Sunyer 2008; Zanchi *et al.* 2008).

Numerous studies (Araujo and Nel 2009; deHaar *et al.* 2006; Donaldson and Stone 2003; Henning *et al.* 2010; Kreyling *et al.* 2006; Oberdorster *et al.* 2002a; Oberdorster *et al.* 1994; Oberdorster 1996; Oberdorster *et al.* 2000; Pedata *et al.* 2010; Schmid *et al.* 2009; Terzano *et al.* 2010; Valavanidis *et al.* 2008) have reported that the cardiopulmonary mortality and morbidity associated with PM exposure appears to be inversely related to particle size, with ultrafine particles being more inflammatory and toxic than larger size PM. The current data, using cultures of nerve cells support this relationship. The reason for this size-dependent toxicity may be physiological and/or physicochemical. Most of the largest size PM particles are either swallowed or trapped by the nose hairs. Dosimetry studies indicate that those particles that physically enter the lungs deposit at different levels of the pulmonary lobes, depending on their size (Phalen and Mendez 2009). The inhaled ultrafine particles deposit to the lower pulmonary lobes where they have a longer retention time in the lung parenchyma and a lower clearance rate (Henning *et al.* 2010; Oberdorster *et al.* 1994; Pedata *et al.* 2010). The physical dimensions of the ultrafine particle gives it an exponentially higher “surface area to mass ratio” relative to larger size particles which allows them to accumulate and deliver higher “loads” of biocontaminants to target cells (Araujo and Nel 2009; Donaldson *et al.* 1996; Donaldson and Stone 2003; Kreyling *et al.* 2004; Kreyling *et al.* 2006; Phalen and Mendez 2009; Schmid *et al.* 2009).

Numerous studies have associated oxidative stress with particle toxicity (Allen 2008; Araujo and Nel 2009; Donaldson and Stone 2003; Hersoug *et al.* 2011; Li and Nel 2006; MohanKumar *et al.* 2008; Moller *et al.* 2010; Stone *et al.* 2007; Terzano *et al.* 2010; Xia *et al.* 2004; Xia *et al.* 2006; Zanchi *et al.* 2008). The current data indicate that *in vitro*, oxidative stress is not a prerequisite to neurotoxic damage since toxicity was produced in neurons and brain cultures dissociated from normal Sprague-Dawley rats. This study also reports that both AMF and UF samples of ambient PM produce biochemical and morphological evidence of apoptosis in the larger diameter neurons of the striatum. Evidence of apoptosis in human respiratory epithelia exposed to PM (Agopyan *et al.* 2003; Chin *et al.* 1998), in the myelin producing glia of feral dogs exposed to air pollutants in Mexico city (Calderon-Garciduenas *et al.* 2003), biochemical changes in the substantia nigra of animals (Villarreal-Calderon *et al.* 2010) and cellular and genomic expressions in BV2 microglia exposed to CAPs collected from Sterling Forest (Sama *et al.* 2007). Although neuronal death can occur by various morphological and biochemically distinct pathways, apoptosis results from disruption of mitochondrial bioenergetics and is largely an oxidative stress driven event (Chandra *et al.* 2000; Hengartner 2000). As noted in AMF and UF treated striatum, significant neuronal death was observed in the presence of both apoptosis and reactive nitrogen species. These data, coupled with the above literature suggest that microglia, which generate the nitrogen species and mediate oxidative stress in neurodegeneration, play a critical but as yet unexplored role in PM-neurotoxicity.

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Highlights

- This in vitro study examines the response of isolated dopaminergic neurons and brain striatum cultures (Sprague-Dawley rat) to concentrated air pollutants (CAPs).
- Evidence of particle-size dependent neurotoxicity is documented in both in vitro systems.
- Evidence of oxidative stress damage is collected in both systems.
- Neurotoxicity is enhanced in brain striatum cultures relative to isolated neurons exposed to CAPs.
- Unlike in vivo models of CAPs Apo $-/-$ neurodegeneration, oxidative stress is not critical to in vitro neurotoxicity.

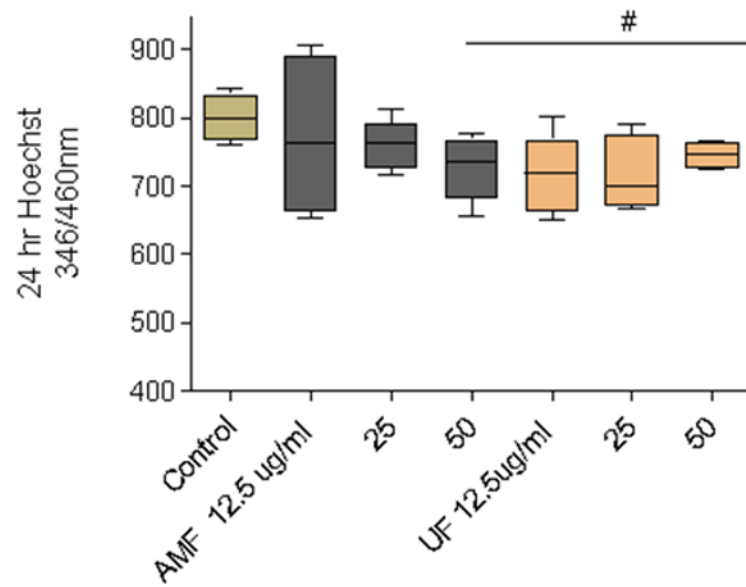


Figure 1.

Loss of nuclear material in N27 isolated dopaminergic neurons was measured using the Hoechst fluorescent probe which binds nuclear adenine-thymine rich regions of double stranded DNA. Cells were exposed to AMF or UF (12.5 $\mu\text{g/ml}$ - 50 $\mu\text{g/ml}$) for 24 hr. Significant cellular decreases were observed in N27 cultures exposed to AMF (50 $\mu\text{g/mL}$) and UF (12.5 $\mu\text{g/ml}$). (#, $p < 0.05$, Dunnett's correction).

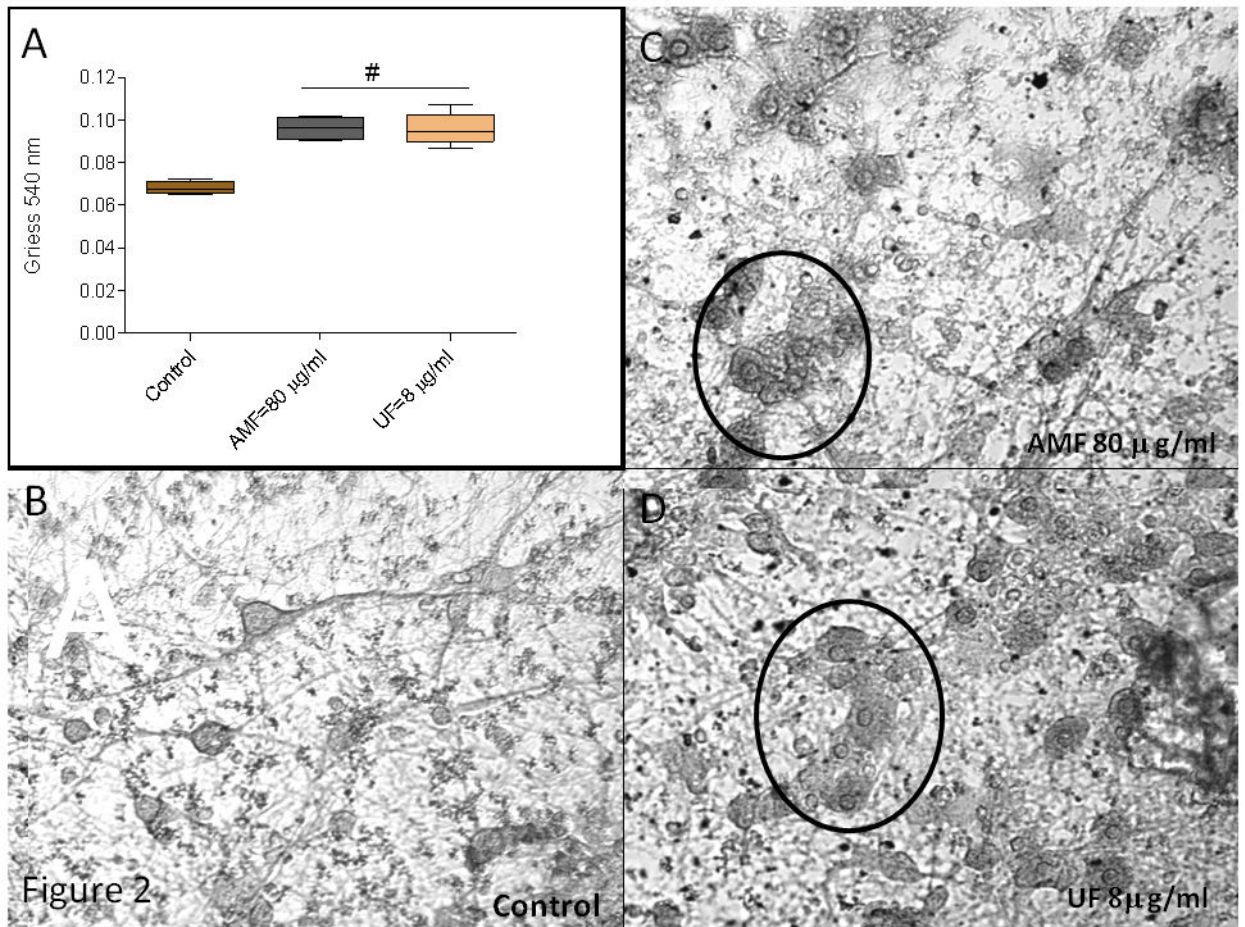


Figure 2.

(A) Microglia-generated nitrite was measured in the supernatant of media or PM exposed striatal cultures using the Griess Reagent. Significant increases over controls were measured in both AMF and UF supernatants (#, $p < 0.01$, one way ANOVA, Dunnett's correction). (B-D) Primary cultures of embryonic rat striatum were immunohistochemically stained with NSE. Microscopic examination indicated that the majority of AMF and UF exposed larger diameter neurons were apoptotic (circles) after 24hr exposure.

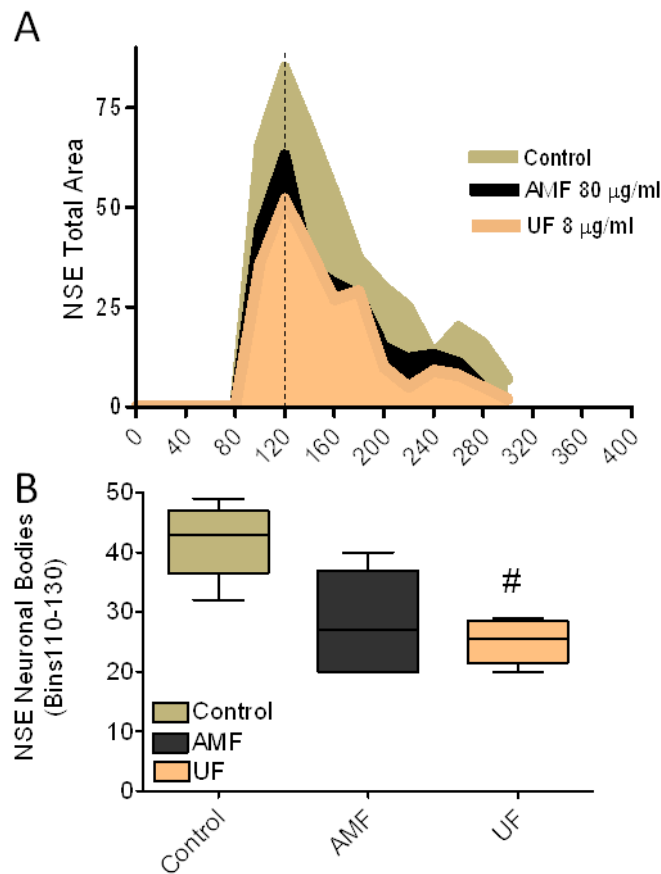


Figure 3.

(A) Morphometric analysis of total NSE stain area (neuron plus axonal plexi) indicated peak reductions in bins 110-130 in cultures treated with either AMF or UF particles, relative to controls after 24 hr. (B) Parameters which described the neuronal (cell body alone) population were used to measure NSE staining in bins of peak reduction. Only the UF treated cultures showed significant reduction of stain (#, $p < 0.05$, One way ANOVA, Dunnett's p corrected) relative to controls. Photos were shot using a 10 \times apocromatic lens on a Nikon TE300 inverted microscope fitted with a cooled-frame CCD camera (Orca I, Hamamatsu, Inc.). Each digitized image was analyzed using Metamorph 7.0 software (Molecular Device, Sunnyvale, CA).