Journal of Alzheimer's Disease 47 (2015) 49–54
DOI 10.3233/JAD-150099
IOS Press

Short Communication

Additive Impairment of Synaptic Signaling in Cultured Cortical Neurons by Exogenously-Applied Oligomerized Amyloid-β and Airborne Nanoparticles Generated during Photocopying

Sangmook Lee[a,c], Ikjot S. Sohal[a,e], Mikaela A. Therrien[a,c], Anoop K. Pal[d,e], Dhimiter Bello[b,d] and Thomas B. Shea[a,c,*]
[a]*Laboratory for Neuroscience, University of Massachusetts Lowell, Lowell, MA, USA*
[b]*Nanomanufacturing Center for Excellence, University of Massachusetts Lowell, Lowell, MA, USA*
[c]*Departments of Biological Sciences, University of Massachusetts Lowell, Lowell, MA, USA*
[d]*Departments of Work Environment, University of Massachusetts Lowell, Lowell, MA, USA*
[e]*Biomedical Engineering and Biotechnology Program, University of Massachusetts Lowell, Lowell, MA, USA*

Accepted 7 April 2015

Abstract. Photocopying in offices and printing centers releases nanoparticles that can reach the brain following inhalation. We examined whether subcytotoxic levels of airborne photocopy-emitted nanoparticles could potentiate perturbation of synaptic signaling in cultured neurons following exposure to amyloid-β (Aβ). Signaling was only transiently inhibited by Aβ or nanoparticles individually, but remained statistically reduced in cultures receiving both after 24 h. *In vitro* and *in vivo* studies with copier emitted nanoparticles have consistently demonstrated inflammation, oxidative stress, and cytotoxicity. Since Aβ can accumulate years before cognitive decline, subcytotoxic levels of nanoparticles are one factor that could potentiate Aβ-induced impairment of synaptic activity during these early stages.

Keywords: Amyloid-β, multi-electrode arrays, nanoparticles, signaling, neurotoxicology

One hallmark of Alzheimer's disease (AD) is the deposition of so-called "senile plaques" in brain tissue [1], of which amyloid-β (Aβ) is a major component. Prior to coalescence into plaques, Aβ oligomers exacerbate oxidative damage in vulnerable neurons, leading to inflammation and cell death [2, 3]. Resultant oxidative stress may further facilitate Aβ production and aggregation, leading to a deleterious neurodegenerative cycle [4].

Aβ can be detected in brain years to decades before overt cognitive decline, suggesting that the above Aβ-induced oxidative damage cycle may precede overt AD pathology [3]. In this regard, we recently demonstrated that subcytotoxic levels of Aβ oligomers impaired synaptic signaling within hours, leaving open the possibility that minor cognitive impairments, not

*Correspondence to: Thomas B. Shea, University of Massachusetts Lowell, 1 University Avenue, Lowell, MA 01821, USA, Tel.: +1 978 934 2881; Fax: +1 978 934 3044; E-mail: thomas_shea@uml.edu.

detectable by standard cognitive tests, could accompany early, subclinical accumulation of Aβ in human brain [5]. We further demonstrated that physiological levels of iron potentiated the impact of Aβ [6]. This interaction highlighted the potential deleterious impact of otherwise benign levels of common environmental compounds on neurodegeneration resulting from early accumulation of Aβ.

Neurotoxicity of engineered nanoparticles is of great interest; yet this remains an understudied area of nanotoxicology. Deposition of airborne nanoparticles in the nose and upper airways is particularly relevant to neurotoxicology, as they may reach the brain via the olfactory nerves as well as following inhalation into the lungs [7, 8], leading to potential disruption of synaptic signaling between the neurons

Photocopiers and printers emit nanoparticles, leading to indoor environments with potentially high airborne nanoparticle exposures. These nanoparticles are a complex mixture of carbonyl rich organic constituents and several metal oxide nanoparticles, added as performance enhancers in toners. Iron oxide, titania, alumina, amorphous silica, copper oxide, possibly zinc oxide, and smaller amounts of manganese and other transition metals have been documented in toners and airborne nanoparticles [9, 10]. This mixture of carbonyl rich organics and redox-active transition metals may contribute synergistically to the high oxidative stress and inflammation documented in human volunteers, chronically exposed workers, and *in vitro*, in several recent studies [11–13].

Herein, we examined whether or not subcytotoxic levels of nanoparticles from a photocopy center could potentiate the deleterious impact of subcytotoxic levels of Aβ on synaptic signaling in murine cortical neuronal cultures.

C57BL/6 murine embryonic cortical neurons harvested at day 17 of gestation were cultured for 4–6 weeks before use in serum-free Neurobasal medium containing 2 mM l-glutamine and antibiotics/antimycotics on poly-D-lysine/fibronectin-coated petri dishes containing multi-electrode arrays (Multichannel Systems, Reutlingen, Germany) [14]. Cultures in each experiment were established from homogenates obtained from multiple embryos from the same pregnant mouse. Multiple pregnant mice were utilized for experimental repetitions and mice were sacrificed according to procedures approved by our Institutional Animal Care and Use Committee. Cultures were incubated for 1 month prior to treatment, during which time they establish a stable network. This is characterized by a transition of predominantly high-amplitude individual signals to a pattern of complex bursts that radiate throughout the network [14, 15].

Human Aβ 1-42 (rPeptide, Bogart GA) was dissolved in HPLC-grade water at 6 mg/mL, diluted to 1 mg/mL in calcium- and magnesium-free phosphate-buffered saline and incubated for 24 h at 37°C for oligomerization [4].

Copier-emitted nanoparticles were collected from ambient air during the printing process in a photocopy center using the Harvard compact cascade impactor as described [9]. Only the nanosize fraction (particulate matter <100 nm, or $PM_{0.1}$ collected on a Teflon filter was used herein. Nanoparticles were extracted from the Teflon filter using sonication, followed by subsequent preparation of dispersions using a standardized dispersion and characterization protocols as described [11], which included determination of the critical sonication energy, agglomerate size, and dispersion stability. The effective density of particle agglomerates in cell culture suspension was measured using the volumetric centrifugation method of Cohen et al. [15]. The effective dose of nanoparticles delivered to cells was computed using advanced fate and transport models. Independent characterization of nanoparticles dispersed in neurobasal medium revealed similar particle properties as previously reported with an estimated 20% of particles deposited on cells by 24 h [11].

Stock nanoparticle dispersion was prepared at 1 mM (1 mg/mL) in deionization H_2O. Nanoparticles were further diluted to give a final administered dose of 0.1–5-μg/mL in supplemented neurobasal medium (delivered dose ~0.02–10 μg/mL at 24 h).

Culture medium was replaced with fresh medium 24 h prior to treatments. Baseline signals were recorded, after which nanoparticles and/or Aβ were aliquoted into the culture medium. Signals were recorded at baseline and after 2, 4, and 24 h.

Signals were recorded over 30-s intervals at room temperature in a MEA-1060-INV amplifier (Multichannel Systems) interfaced with a DT9814 data acquisition system (Data Translation, Marlborough, MA) [13]. Signals were classified as spikes (individual signals deviating from baseline by >0.0025V) or bursts (≥3 consecutive signals deviating from the baseline by >0.0025V prior to decreasing to <0.0025V) Prior studies confirmed these signals to be synaptic in origin [13]. Total signals (all spikes plus all bursts exceeding 0.0025V) were quantified via our MEA software. The entire acquisition process, from removal of a culture from the incubator until its return took <2 min; accordingly no perfusion of cultures was warranted,

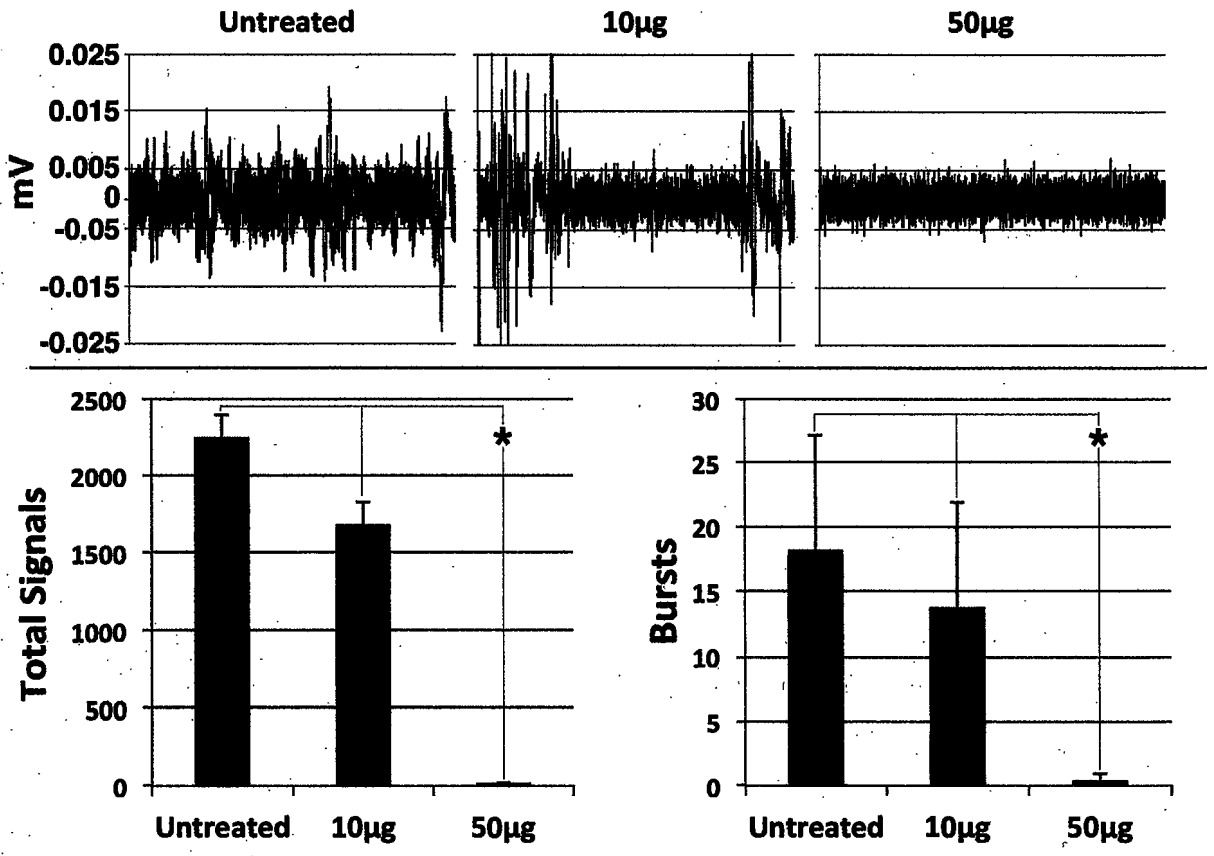

Fig. 1. Concentration-dependent inhibition of synaptic signaling by copier-emitted nanoparticles. Panels present 20-s excerpts from spontaneous signals recorded from representative neuronal cultures 24 h after addition of 10 or 50 μg of nanoparticles, along with untreated cultures as indicated. The accompanying graphs present quantification of total signals and bursts after 24 h of treatment. Values represent the mean ± standard deviation of Volts × 10^{-1} derived from at least three recordings from each of two cultures.

and signals were recorded at ambient room temperature. Each culture was treated identically. Data files for each channel were analyzed manually and with an algorithm that distinguishes neuronal activity from baseline electrical disturbances [16]. Bursts were identified by visual inspection of signals recorded over 30-s intervals at each sampling interval [13, 14]. Values were derived from duplicate cultures from two experiments, for a total of four cultures under each condition.

Signals of untreated cultures and all treatments were analyzed by ANOVA with Fischer's *post-hoc* analyses 24 h after treatment. For clarity of presentation in some graphs, the mean of baseline signals from all cultures prior to treatment was defined as 100%, and the percentage of signals at each sampling interval for each condition was calculated [4, 5].

Treatment of cultures with 50 μg/mL nanoparticles reduced total signaling and complex bursts by >98% within 24 h. By contrast, treatment with 10 μg/mL reduced total signaling and complex bursts by approx. 25%, which was significantly identical to values displayed by cultures not treated with nanoparticles (Fig. 1). We therefore utilized 10 μg/mL copier nanoparticles for further analyses.

As shown previously, treatment with 10 nM Aβ induced a transient decrease in complex bursts (Fig. 2). However, combined treatment with Aβ plus 10 nM nanoparticles progressively reduced bursts such that after 24 h they remained statistically reduced versus untreated cultures. No change was observed in the number of total signals, confirming an absence of overall toxicity after 24 h (Fig. 1).

Analysis of signaling affords visualization of neurotoxic impact not detectable by conventional cytotoxicity analyses. These findings demonstrate that exposure to sub-cytotoxic concentrations of copier-emitted nanoparticles and Aβ significantly reduced synaptic signaling at concentrations at which neither

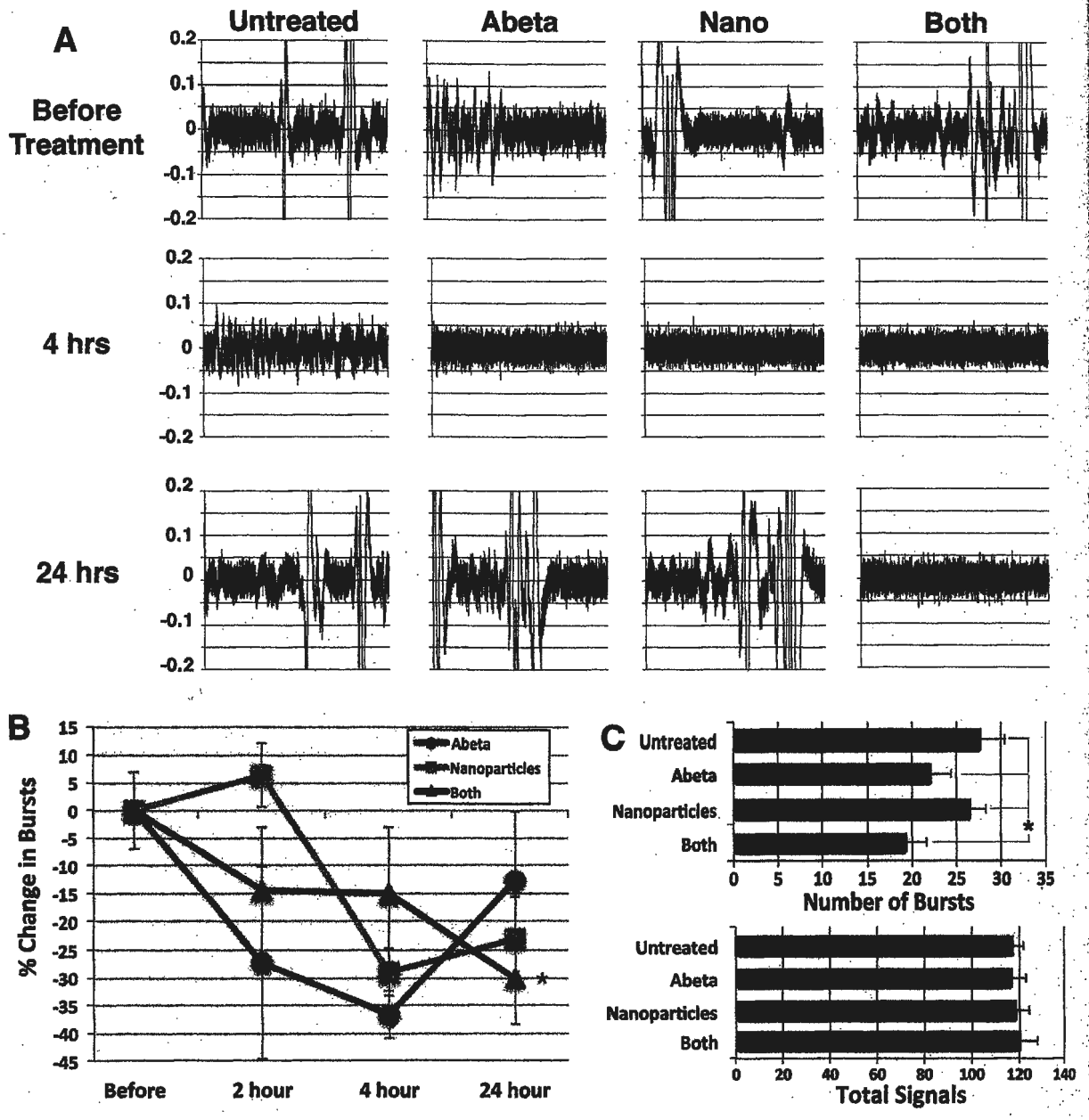

Fig. 2. Exposure to Aβ and nanoparticles inhibited synaptic signaling in cultured cortical neurons. A) 15-s excerpts from spontaneous signals recorded from neuronal cultures before and at 4 and 24 h following addition of Aβ, copier nanoparticles, or both, along with untreated cultures, as described in the text. Signal amplitude is indicated in volts $\times 10^{-1}$. The same cultures are presented at each sampling interval. Note the transient decline in signals in cultures treated with Aβ or nanoparticles, followed by recovery at 24 h. By contrast, signals remain inhibited at 24 h in cultures treated with both Aβ and nanoparticles. B) Quantification of positive bursts >0.0025V from multiple cultures as presented in (A). Values represent the mean change in bursts (±standard deviation from the mean) recorded under all conditions versus those recorded in untreated cultures as described in the text. Bursts underwent an initial decline following treatment with either Aβ or nanoparticles, but had undergone recovery by 24 h and were statistically identical to untreated cultures. Bursts were statistically reduced in cultures receiving both Aβ and nanoparticles versus untreated cultures ($^*p<0.05$, ANOVA with Fischer LSD *post-hoc* analyses). C) Graphs present comparison of bursts and total signals ($\times 10^{-3}$) as indicated at 24 h after treatment; bursts were statistically reduced (in cultures receiving both Aβ and nanoparticles versus untreated cultures ($^*p<0.05$, ANOVA with Fischer LSD *post-hoc* analyses). No change was observed in total signals.

agent alone only transiently affected signaling. These studies extend our prior investigations, where a higher but still sub-cytotoxic concentration of Aβ (1 μM) induced signaling abnormalities within hours when administered in isolation [4], and simultaneous exposure to physiological concentration of iron potentiated Aβ-induced signaling abnormalities [6]. Herein, and in our prior studies, signals reverted to baseline levels within 24 h following treatment with Aβ alone, indicating that exposure to a single subcytotoxic dosage of Aβ did not invoke permanent damage. One potential contributing factor for restoration of normal signaling is the internalization of Aβ by neurons and/or glia in these cultures as described previously [17].

Aβ can be detected years prior to any overt impairment of cognitive performance [3]. Under such conditions, individuals harboring aberrant Aβ accumulation will likely continue their prior day-to-day activities. Some occupations demand full time working with printers or copiers. Engineered nanomaterials are usually incorporated in toners but emitted nanoparticles have a complex chemistry reflecting both toner and paper [10]. Recent research on photocopiers and printers has highlighted these devices as nano-enabled products and potential source of indoor air pollutants, with release of nanoparticles of complex chemistry-containing both organic and inorganic phases. Previously, systemic oxidative stress and upper airway inflammation in healthy individuals was reported in healthy individuals following a single day exposure in a photocopy center environment [18]. Nanoparticles emitted by copiers induce lung injury and inflammation in mice [12]. Extensive physicochemical and morphological characterization of emitted nanoparticles has shown the particles to be a mix of various metal/metal oxides [10]. The potential synergism in reactive oxygen species generation between organic and inorganic constituents of complex mixtures is well documented and likely at play with copier emitted nanoparticles.

The impact of nanoparticles plus Aβ on signaling may be minor enough to remain undetected but could nevertheless compromise and/or delay decision-making under critical/stressful conditions. Notably, humans are exposed to nanoparticles comprised of silver copper and/or aluminum at a number of manufacturing sites. Such nanoparticles induced developmental and age-related sensory, motor, and cognitive compromises in rodent studies; of interest will be to examine the impact of such additional environmental nanoparticles on Aβ toxicity.

ACKNOWLEDGMENTS

This research was funded by US Army Research Office W911NF1110125 and Grant No. 2 T42 OH008416-09, from the National Institute for Occupational Safety and Health (NIOSH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIOSH.

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/15-0099r2).

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