Pyroglutamate-modified amyloid-β (pE-Aβ) is a highly neurotoxic amyloid-β (Aβ) isoform and is enriched in the brains of individuals with Alzheimer disease compared with healthy aged controls. Pyroglutamate formation increases the rate of Aβ oligomerization and alters the interactions of Aβ with Cu^{2+} and lipids; however, a link between these properties and the toxicity of pE-Aβ peptides has not been established. We report here that Aβ3pE-42 has an enhanced capacity to cause lipid peroxidation in primary cortical mouse neurons compared with the full-length isoform (Aβ1–42). In contrast, Aβ1–42 caused a significant elevation in cytosolic reactive oxygen species, whereas Aβ3pE-42 did not. We also report that Aβ3pE-42 preferentially associates with neuronal membranes and triggers Ca^{2+} influx that can be partially blocked by the N-methyl-d-aspartate receptor antagonist MK-801. Aβ3pE-42 further caused a loss of plasma membrane integrity and remained bound to neurons at significantly higher levels than Aβ1–42 over extended incubations. Pyroglutamate formation was additionally found to increase the relative efficiency of Aβ-dityrosine oligomer formation mediated by copper-redox cycling.

Aβ3 peptides are found in every human brain; however, the concentration and composition of Aβ peptide isoforms are distinctly different in healthy individuals and people with AD (1–3). Amino-truncated Aβ peptides are abundant in the AD brain (4, 5) and increase in prevalence with disease progression (6). The process of Aβ amino-truncation can occur via the actions of aminopeptidases on full-length Aβ peptides (7, 8), via altered cleavage of amyloid precursor protein in the generation of Aβ (9–11), and potentially by Aβ-copper-redox cycling reactions (12). As a consequence, amino-truncation can expose glutamate residues (positions 3 and 11 of Aβ) to cyclization by the action of glutaminyl cyclase (QC), forming the highly amyloidogenic pyroglutamate-Aβ (pE-Aβ) peptides Aβ3pE-40, Aβ3pE-42, Aβ11pE-40, and Aβ11pE-42 (7, 13).

Pyroglutamate formation significantly increases the hydrophobicity of Aβ, causing the peptide to aggregate more rapidly and form oligomers at lower concentration thresholds (5, 14, 15). pE-Aβ peptides also demonstrate increased β-sheet (aggregate structure) stability (16, 17), differences in fibril ultrastructure (18, 19), and altered interactions with copper ions (20, 21) and synthetic lipid membranes (22, 23). Notably, trace quantities of Aβ3pE-42 have been observed to dramatically enhance the aggregation and neurotoxicity of Aβ1–42 (24), promoting descriptions of pE-Aβ as “prion-like.” Still, it remains unclear as to the cytotoxic potency of pE-Aβ peptides compared with their full-length Aβ counterparts. Some studies have demonstrated pE-Aβ peptides to have enhanced toxicity (24–26), although others have reported no difference in toxicity between the isoforms (27–30). Methodological differences may account somewhat for variability in the relative toxicities reported (Table 1), yet molecular mechanisms to explain changes in cytotoxicity have not been defined.

One mechanism through which Aβ peptides cause cytotoxicity is by production of reactive oxygen species (ROS) via facile copper-redox cycling (31–33), which can in turn effect oxidative damage to neuronal proteins and lipids (34). Imbalances in ROS production and detoxification are strongly implicated in AD neurodegeneration, reflected by cerebral elevations in oxidized DNA, lipids, and proteins (35–37). Pyroglutamate formation alters Aβ-Cu^{2+} coordination modes (20, 21), although it is not known whether this affects the capacity of pE-Aβ peptides to undertake redox cycling and produce cytotoxic ROS. We therefore aimed to determine whether full-length Aβ and pE-Aβ possess differences in their capacity to alter ROS flux and cause oxidative damage to neurons in vitro. Additionally, Aβ isoforms were compared for their capacity to form oligomers and covalent tyrosine-tyrosine bonds (dityrosine) as a result of Aβ-copper-redox cycling. The capacity for Aβ to form dityrosine has previously been correlated with neurotoxicity (38), although recent reports have found that Aβ fibrils within...
amyloid plaques contain intense dityrosine immunoreactivity (39), indicating that dityrosine formation may be associated with AD amyloidogenesis. Further comparisons were made between the peptides for their capacity to perturb neuronal membranes and induce changes in neuronal ion homeostasis (Ca\(^{2+}\)/H\(_{11001}\) flux).

**Experimental Procedures**

**Materials**—A\(\beta\) peptides were purchased from the ERI Amyloid Laboratory, LLC, and purified by reversed-phase HPLC to >95% purity. All chemicals used were analytical grade and purchased from ChemSupply (Australia) unless otherwise stated. Thioflavin-T, coumarin-3-carboxylic acid (3-CCA), butylated hydroxytoluene, bovine serum albumin, and paraformaldehyde were purchased from Sigma. Diamin (1,8-diaminonaphthacamine) was synthesized as reported previously (40).

**Preparation of A\(\beta\) Solutions and Cu\(^{2+}\)-oxidized A\(\beta\) Oligomers**—A\(\beta\) stock solutions were prepared by dissolving lyophilized peptides to 5 mg/ml in NaOH (60 mM) and incubating at ambient temperature for 5 min to dissociate aggregated material. Solutions were then diluted to 1 mg/ml in MilliQ H\(_2\)O and 10X PBS (PBS is defined as 50 mM sodium phosphate, 2.7 mM KCl, 137 mM NaCl) at a v/v/v ratio of 2:7:1 (NaOH, H\(_2\)O, 10X PBS). The preparation was sonicated for 10 min in an iced water bath and then centrifuged at 16,500 \(\times\) g for 10 min at 4 \(^\circ\)C. Supernatants (upper 80% of solution) were removed to pre-chilled tubes on ice; pH was adjusted by addition of NaH\(_2\)PO\(_4\) (0.5 M) to 1.0% v/v and then kept on ice for immediate use. A\(\beta\) stock concentrations were determined by UV spectrometry by measuring absorbance at 214 nm (A\(_{214}\)) and applying the following extinction coefficients (M\(^{-1}\) cm\(^{-1}\)) determined from UV scans and amino acid analysis: A\(\beta\)(1–40) = 91,462; A\(\beta\)(3pE-40) = 89,705; A\(\beta\)(1–42) = 94,526; and A\(\beta\)(3pE-42) = 90,925.

A\(\beta\) oligomers were generated by reacting A\(\beta\) (10 \(\mu\)M) with Cu\(^{2+}\) (10 \(\mu\)M, as CuCl\(_2\) glycine\(_6\)) and ascorbate (100 \(\mu\)M) at 37 \(^\circ\)C on a vertically rotating wheel at 20 rpm, in 2-ml round-bottom tubes (catalog no. 0030123.344, Eppendorf). Reactions were halted with the addition of EDTA (to 250 \(\mu\)M) and placed on ice. Control incubations of A\(\beta\) only were incubated and sampled under identical conditions for comparison. A\(\beta\) fibrillation assays (thioflavin-T assays) were performed as described previously (41).

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**TABLE 1**

Overview of publications comparing the cytotoxicity of pE-A\(\beta\) and full-length A\(\beta\) peptides in vitro

The following abbreviations are used in table: CSF, cerebrospinal fluid; DMSO, dimethyl sulphoxide; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; LDH, lactate dehydrogenase; PBS, phosphatebuffered saline.

<table>
<thead>
<tr>
<th>Reference</th>
<th>A(\beta) Stock Preparation</th>
<th>A(\beta) Treatment</th>
<th>Toxicity Measurements</th>
<th>Results</th>
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<td>Tekirian et al., 1999 (27)</td>
<td>Lyophilized in 1 mM HCl then redissolved in cell culture media or PBS (pH 7.4)</td>
<td>Fresh peptides dissolved in cell culture media to 5 – 50 (\mu)M</td>
<td>Phase-contrast micrographs of primary cortical rat neurons taken after 6, 3, 6, 12 and 24 h treatment. Cells that were smooth, round and vacuole free scored as ‘alive’.</td>
<td>No difference in toxicity between A(\beta)-40 and A(\beta)3pE-40, nor A(\beta)-42 and A(\beta)3pE-42 at any time-point, freeze or aged.</td>
</tr>
<tr>
<td>Russe et al., 2002 (49)</td>
<td>DMSO, 1 mM A(\beta) stock</td>
<td>A(\beta) oligomerized for 3 days (37 (^\circ)C) in cell media and applied to cells at 0.1, 1.0 or 10 (\mu)M for 24 h.</td>
<td>Cortical autolysos and hippocampal neurons viability measured by mitochondrial function (MTT assay) and cell permeability (LDH leakage).</td>
<td>A(\beta)3pE-40 more toxic than all other peptides on astrocytes (MTT assay), no difference between other peptides. A(\beta)3pE-40 &amp; A(\beta)3pE-42 induce more LDH leakage from astrocytes than A(\beta)-40 &amp; A(\beta)-42 - no differences for neurons.</td>
</tr>
<tr>
<td>Shiratori et al., 2002 (28)</td>
<td>Expression of APP constructs to produce different A(\beta) isoforms in primary cortical mouse neurons</td>
<td>A(\beta) peptides incubated in cell culture media for 1 h (room temperature). Incubated on cells at 1 (\mu)M (24 – 48 h) or injected in brains (500 pmol total, for 2 – 14 days).</td>
<td>Primary cortical mouse neurons viability measured by caspase induction, MTT and calcein assays. ROS measured by dichlorofluorescin assay 2 days post-injections, behavior day 14.</td>
<td>Same decrease in cell viability between all constructs compared to control after 3 days of expression. No difference in induction to H(_2)O; or levels of Calpain activation after APP constructs.</td>
</tr>
<tr>
<td>Youssef et al., 2008 (29)</td>
<td>Peptides dissolved to 5 mg/ml in HEPES, dried, then redissolved in cell culture media.</td>
<td>A(\beta) peptides incubated in cell culture media for 1 h (room temperature). Incubated on cells at 1 (\mu)M (24 – 48 h) or injected in brains (500 pmol total, for 2 – 14 days).</td>
<td>No difference in toxicity between all constructs compared to control after 3 days of expression. No difference in induction to H(_2)O; or levels of Calpain activation after APP constructs.</td>
<td></td>
</tr>
<tr>
<td>Nussbaum et al., 2012 (24)</td>
<td>A(\beta) dissolved in HEPES, lyophilized by vacuum centrifugation then reconstituted in 1% NH(_4)OH and diluted in PBS</td>
<td>A(\beta) (5 (\mu)M) in culture media incubated 24 h at 4 (^\circ)C, then 24 h on cells at (\leq) 1 (\mu)M.</td>
<td>A(\beta)3pE-42 &amp; A(\beta)-42 made from peptides either incubated together, or separately then mixed, before applying to cells.</td>
<td>Primary cortical mouse neuron viability measured by caspase induction, MTT assay and calcein-AM vital stain.</td>
</tr>
<tr>
<td>Galante et al., 2012 (25)</td>
<td>A(\beta) dissolved in DMSO, lyophilized by vacuum centrifugation then reconstituted in 1% NH(_4)OH and diluted in PBS</td>
<td>Peptides separated into small (4.5 kDa), medium (14 kDa) and large (99 kDa) aggregates, then incubated on cells at 100 (\mu)M for up to 4 days</td>
<td>SII-SYS human neuroblastoma viability measured by MTT assay and xCELLigence (Roche) real-time viability monitoring.</td>
<td>Medium and large aggregates of both A(\beta)-42 and A(\beta)-3pE-42 were significantly toxic to neurons, however small A(\beta)-42 small aggregates were additionally toxic while A(\beta)-42 small aggregates were not.</td>
</tr>
<tr>
<td>Schlenzig et al., 2012 (26)</td>
<td>A(\beta) dissolved in HEPES, dried by evaporation then redissolved in DMSO.</td>
<td>HEPES treated peptides re-dissolved to 30 (\mu)M in DMSO then diluted in artificial-CSF to 250 or 500 (\mu)M and incubated on hippocampal slices.</td>
<td>Mouse hippocampal slice long-term potentiation (LTP) measured following 2 h exposure to synthetic peptides in rat hippocampal artificial-CSF.</td>
<td>A(\beta)3pE-42 significantly more inhibitory to LTP than A(\beta)-40 at 500 (\mu)M. Likewise A(\beta)-3pE-42 significantly more inhibitory than A(\beta)-42 at 250 (\mu)M.</td>
</tr>
<tr>
<td>Bouter et al., 2013 (30)</td>
<td>A(\beta) dissolved in HEPES, frozen, then HEPES evaporated. Sticks dissolved in NaOH (100 mM) to 2 mg/ml, stored at -80 (^\circ)C until use.</td>
<td>A(\beta) stocks freshly diluted to 1, 5 or 10 (\mu)M in DMEM/F12 media and applied to cells for cultures at 24 h.</td>
<td>Cortical rat neurons cultured in 48-well plates, viability measured by calcein-AM vital stain using fluorescence plate reader.</td>
<td>Identical toxicity observed for A(\beta)-42, A(\beta)-3pE-42 and A(\beta)-42 at all concentrations tested.</td>
</tr>
</tbody>
</table>
Aβ-Dityrosine Determination and Hydrophobic Index Calculation—The dityrosine content of Cu2+-reacted Aβ samples was determined by fluorescence spectrophotometry (λ 320 nm excitation and λ 420 nm emission), as described previously (43). Reaction half-times and rate constants (K = min−1) were calculated from one-phase association regressions of fluorescence data (GraphPad Prism version 6.0). Calculation of Aβ hydrophobic scores were determined from the sum of the amino acid residue hydrophatic indexes (44). Pyroglytamy residues were assigned a hydropathy value of −1.0.

Aβ Detection by Western Blot—Tissue extracts and synthetic peptides were separated by SDS-PAGE using 4–12% XT Bis-Tris gels (Criterion, Bio-Rad) according to the manufacturer’s instructions. Samples were transferred to pre-assembled PVDF membrane stacks using a Trans-Blot® semi-dry transfer apparatus (Bio-Rad). Blots were blocked in TBS-T (10 mM Tris-HCl, 50 mM NaCl, 0.1% v/v Tween 20, pH 8.0) containing 5% w/v skim milk. Primary antibodies were incubated on blots for at least 1 h at room temperature or overnight at 4 °C. HRP-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulins (Dako) were diluted 1:10,000 in TBST and incubated for 1 h at room temperature. All antibodies were diluted in TBS-T containing 5% skim milk and 0.05% w/v sodium azide. Blots were washed four times for 10 min in TBS-T after each primary and secondary antibody binding step. Chemiluminescence signals were captured after application of ECL (Immobilon, Millipore) with an LAS3000 detector and analyzed using MultiGauge software (Fujifilm). Aβ peptides were detected using the monoclonal mouse antibodies 4G8 (Covance) or 6E10 (Signet Laboratories) diluted to 1 μg/mL. The dityrosine modification was detected using the 1C3 antibody raised against synthetic dityrosine (catalog no. NWA-DIY020, Northwest Life Science Specialties) at a 1:1000 dilution.

Size-exclusion Chromatography (SEC) and Atomic Force Microscopy (AFM)—Aβ oligomers in HBSS buffer were prepared as above but at twice the concentration (20 μM Aβ, 20 μM Cu2+, 200 μM ascorbate) to provide adequate signals for measurement.

SEC analysis was performed using a BioLogic DuoFlow QuadTec 40 system (Bio-Rad) fitted with a Superdex 75 10/300 column (catalog no. 17-5174-01, GE Healthcare). Both equilibration and operation of the column were in Tris-buffered de-gassed) at a flow rate of 0.5 ml/min and ambient temperature. The absorbance at 214, 260, and 280 nm was monitored, collecting 5 data points/s. Samples were injected onto the column (0.5 ml per run, ~45 μg of Aβ) immediately after collection at indicated time points.

AFM analyses were performed on Aβ reactions prepared in Neurobasal medium (catalog no. 21103-049, Gibco) at 10 μM. Samples (10 μl) were collected and spotted on freshly cleaved mica, dried at room temperature for 5 min in a laminar flow hood, and rinsed with 2 ml of de-ionized water (Milli-Q, Millipore). The sample was blown dry with nitrogen (Coregas Nitrogen 4.0) before being transferred to the AFM sample stage (Asylum Research Cypher AFM). Images were acquired in alternating current (tapping) mode in air using Tap300-G silicon AFM probes (Budget Sensors) with scan rates of 1.5–2.5 Hz; drive amplitude was kept to a minimum with amplitude set-points of 60–80%. The mask threshold was set to 250 pm for image analysis.

Primary Neuronal Cell Culture and Aβ Clearance Assays—All experiments involving animals were conducted in accordance with the Australian Code of Practice for the Use of Laboratory Animals and were approved by the Institutional Animal Experimentation Ethics Committee.

Mouse cortical neuronal cultures isolated from C57Bl/6 E14 embryos were prepared as described previously (45). Cells were plated in poly-D-lysine-coated 48- or 96-well plates at a density of 150,000 cells/cm2. All cell culture materials were purchased from Gibco/Thermo Fisher unless otherwise stated. Cells were grown in Neurobasal medium (NB) containing B27 supplements, gentamicin, and 0.5 mM GlutaMAX™. Fresh NB medium containing B27 minus antioxidants (B27-AO) and cytosine-β-d-arabinofuranoside (2 μM; catalog no. C1768, Sigma) were applied after 6 days in vitro (DIV). Neurons were further incubated until DIV 8 or 9 prior to applying treatments.

Levels of cell-bound Aβ were measured following exposure to Aβ peptides applied in NB medium containing B27-AO and cytosine-β-d-arabinofuranoside. Cells (DIV 8 or 9) were treated for 48 h with Aβ mixtures (10 μM total). The media were removed, and cells were washed twice with Dulbecco’s PBS (catalog no. 14190-144, Gibco), and then the cells were extracted with M-PER reagent (catalog no. 78501, Thermo-Scientific). For fractionation studies, cells were scrapped into TBS, pH 7.5, containing protease inhibitors (complete, catalog no. 11873580001, Roche Applied Science) and probe-sonicated by two rounds of 10 bursts (40% power, 0.5 s each) with a Sonifier S-250D (Branson). Lysates were centrifuged at 100,000 × g, and supernatants (TBS phase) were collected to fresh tubes. The pellets were extracted with an equal volume of Na2CO3 (100 mM, pH 12) for 1 h of incubation on ice and then briefly vortexed and centrifuged again to collect supernatants (carbonate phase). Pellets were resuspended in an equal volume of urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 50 mM Tris, pH 8.0). Western blots were performed on 5 μg of total protein based on BCA assays (catalog no. 23225, Thermo-Scientific) of TBS phases, loading equal volumes for carbonate and urea phases.

Cell Viability Measurement—M17 neuroblastomas were cultured in DMEM containing fetal bovine serum (FBS; 10% v/v) and penicillin/streptomycin (catalog no. 15140, Gibco). Cells were plated in 6-well culture plates (catalog no. 140675, Nunc) at 40,000 cells/cm2 and incubated overnight (37 °C, 5% CO2). Fresh medium supplemented with retinoic acid (10 μM) was applied, and the cells were allowed to differentiate for 48 h. Freshly prepared Aβ solutions (10 μM) or synthetic lipid peroxide (1 μM) were applied to cells for 4 h and then the cells were collected using trypsin and resuspended in 1 ml of PBS containing 2% FBS. Propidium iodide was added to a final concentration of 1 μg/ml. Flow cytometric analysis was performed on a Beckman Coulter CyAn ADP analyzer with Summit 4.0.3 acquisition software. Debris and cell aggregates were excluded by gating. Live and dead cells were then identified based on exclusion and inclusion, respectively, of propidium iodide. Analysis of data were performed using FCS Express 4 analysis software.
Detection of Neuronal ROS Flux and Lipid Peroxidation—

Neuronal ROS flux was measured using the redox-sensitive probe 2,7-dichlorofluorescin diacetate (catalog no. D999, Molecular Probes), essentially as described previously (46). Briefly, 2,7-dichlorofluorescin diacetate (100 μM) was applied to cells (DIV 9) in NB medium supplemented with B27-AO (37 °C, 5% CO₂) for 60 min; the media were then removed, and the cells were rinsed with Hank’s balanced salt solution (HBSS; catalog no. 14175-079, Gibco). Freshly prepared Aβ peptides were diluted to 10 μM in HBSS and applied to the neurons for a further 60 min of incubation (37 °C, 5% CO₂, light protected) until reading DCF fluorescence (λ 485 nm excitation, λ 530 nm emission, and λ 515 nm emission cutoff filter) with a Flexstation III spectrophotometer (Molecular Devices) equilibrated to 37 °C.

Lipid peroxidation was measured using the lipid hydroperoxide probe diphényl-1-pyrenylphosphine (DPPP; catalog no. 62237, Cayman Chemicals), with methodology adapted from Ref. 47. DPPP (stock dissolved in N₂-purged DMSO to 25.88 mg/mL) was diluted to 50 μM in NB media and applied to cells (DIV 8–9) in 96-well black-walled microplates (Greiner) and then incubated for 60 min at 37 °C, 5% CO₂ in the dark. The DPPP-containing media were removed; the cells were rinsed twice with HBSS and then equilibrated in HBSS at 37 °C and 5% CO₂ for 30 min before treatment. Freshly prepared Aβ peptides were applied to cells for 4 h until reading fluorescence (λ 340 nm excitation and λ 380 nm emission) with an EnSpire multimode spectrophotometer (PerkinElmer Life Sciences). Positive control wells were treated with a synthetic lipid hydroperoxide standard (catalog no. 705014; Cayman Chemicals) for comparison. Care was taken at all steps to minimize artifactual oxidation of DPPP by reducing exposure to light.

Neuronal Calcium Flux Measurement—Cortical neurons plated at 230,000 cells/cm² in 96-well black-walled microplates (Greiner) were loaded with the fluorescent Ca²⁺ sensor Fluo4-AM (catalog no. F14201, Molecular Probes) at DIV 9 for 30 min at 37 °C, 5% CO₂, and then equilibrated to room temperature for 30 min. Fluorescence (excitation λ 490 nm and emission λ 520 nm) was measured at base line for 10 reads at 27-s intervals followed by a further 10 reads after injection of glutamate or Aβ (final concentrations of 1 and 10 μM, respectively) using a Fluostar plate reader (BMG Labtech). Ca²⁺ flux values (ΔF/F₀) were expressed as the difference between mean baseline and immediately following glutamate or Aβ application. Experiments determining the effect of metal depletion utilized neuronal cultures pre-treated for 1 h with Diamsar (10 μM). To assess the contribution of NMDAR signaling in Aβ-induced Ca²⁺ flux, cultures were pre-treated for 15 min with the NMDAR antagonist MK-801 (1 μM). In separate experiments the supplied assay buffer (HBSS) was replaced with Ca²⁺/Mg²⁺-free HBSS to determine the source of Ca²⁺ entering the cytosol.

Results

Pyroglutamate Formation Alters the Production of ROS and Dityrosine by Aβ—pE-Aβ peptides demonstrated greatly increased fibrillation rates compared with the respective full-length isoforms (Fig. 1A), consistent with previous reports (14, 18). Aβ(1–40), Aβ(1–42), Aβ3p-E-40, and Aβ3p-E-42 were also compared for their capacity to produce ROS upon reaction with Cu²⁺ and ascorbate. The rate of 'OH production was significantly higher for Aβ(1–42) compared with Aβ3p-E-42 (Fig. 1B). In contrast, Aβ3p-E-40 produced more 'OH than Aβ(1–40) and indeed all other Aβ isoforms. The kinetics of 'OH production showed a similar sigmoidal pattern for all peptides with signals reaching plateau after ~20 min.

Differences in redox cycling and 'OH production between Aβ peptides were further studied to compare formation of dityrosine bonds. Dityrosine formation rates partially reflected 'OH production rates whereby Aβ3p-E-40 formed dityrosine more rapidly than Aβ(1–40) (half-times of 5.44 and 33.64 min, respectively), whereas Aβ(1–42) formed dityrosine more rapidly than Aβ3p-E-42 (half-times of 5.38 and 7.31 min, respectively) (Fig. 1, C and D). Total Aβ-dityrosine content, however, did not mirror the levels of 'OH produced by each peptide; Aβ(1–42) formed more than double the amount of dityrosine than all other peptides. A direct relationship was not observed between the dityrosine formation rate and relative Aβ hydrophobicity (Fig. 1E); however, the efficiency of dityrosine formation (calculated as a ratio of dityrosine formed per unit of 'OH generated) was increased for both pE-Aβ peptides relative to their full-length counterparts (Fig. 1F).

Comparison of the Oligomerization of Pyroglutamate-Aβ and Full-length Aβ in the Presence and Absence of Cu²⁺—Previous studies have shown differences between Aβ(1–40) and Aβ(1–42) in oligomerization and dityrosine formation when reacted with Cu²⁺ and biological reductants (48). We compared the profiles of full-length Aβ and pE-Aβ oligomers generated in the presence or absence of Cu²⁺ and ascorbate. Aβ(1–42) and Aβ3p-E-42 rapidly formed SDS-stable oligomers within 5 min of reaction with Cu²⁺, whereas increases in Aβ(1–40) and Aβ3p-E-40 oligomers were observed at 15 min (Fig. 2A). Significant formation of SDS-stable oligomers was not observed for Aβ incubated in the absence of Cu²⁺ and ascorbate over the same time period.

Dityrosine cross-links were also detected in Aβ(1–40) and Aβ3p-E-40 reactions by immunostaining with a dityrosine-specific antibody (1C3), revealing predominantly dimeric and trimeric-sized (~8/12 kDa) oligomers and confirming the enhanced capacity for Aβ3p-E-40 dityrosine formation compared with Aβ(1–40) (Fig. 2B). The 1C3 antibody, however, did not show significant immunoreactivity to Western blots of Cu²⁺-reacted Aβ(1–42) and Aβ3p-E-42 peptides (data not shown).

We additionally examined the oligomer states of Aβ(1–42) and Aβ3p-E-42 in non-denaturing conditions by SEC and AFM. After a 4-h incubation in PBS, the Aβ(1–42) oligomer profile did not change considerably from the freshly prepared solution, resolving predominantly as a single low-mass peak (~14 kDa) by size exclusion, with minor signals corresponding to oligomers of ~40–75 kDa (Fig. 3A). Addition of Cu²⁺ and ascorbate to Aβ(1–42) caused a sizable increase in the abundance of oligomers but did not appear to alter their mass. By comparison, Aβ3p-E-42 ternary states changed considerably during the 4-h incubation, observed as a dramatic loss of low-mass peak (~14 kDa) in either the presence or absence of Cu²⁺ and ascorbate (Fig. 3B). After a 4-h incubation, residual signals remaining in
the Aβ3pE-42 reactions without Cu²⁺ resolved predominantly as an oligomeric peak at ~30–45 kDa. In the Aβ3pE-42 reaction containing Cu²⁺ there was some preservation of the monomeric signal after the 4-h incubation, with an additional polydisperse peak across the 30–75-kDa range.

To simulate Aβ oligomerization in cell culture conditions, reactions were conducted in neurobasal media over an extended incubation period, and the peptide structures were assessed by AFM (Fig. 3C). Little difference in peptide structure was observed during a 48-h incubation of Aβ(1–42) in unsupplemented neurobasal media (maximum z < 1 nm). In the Aβ(1–42) reactions supplemented with Cu²⁺/ascorbate, there was similarly no noticeable change after 4-h incubation, although a large increase in the size and abundance of oligomers was observed after 48 h (maximum z = 4.9 nm). Likewise, Aβ3pE-42 structures did not demonstrate a noticeable size difference over a 48-h incubation in neurobasal media in the absence of supplemental Cu²⁺/ascorbate (maximum z < 1 nm), although the reactions supplemented with Cu²⁺/ascorbate showed a stepwise size increase over the incubation period (maximum z = 0.4, 1.0, and 1.8 nm at 0, 4, and 48 h, respectively).

Aβ3pE-42 Predominantly Associates with Neuronal Membranes and Is Resistant to Clearance—pE-Aβ has been found to resist proteolytic clearance in astrocyte cultures (49, 50). We therefore compared the residual levels of Aβ(1–42) and Aβ3pE-42 following extended exposures to cortical neuron cultures, additionally assessing the capacity for minor quantities of Aβ3pE-42 to affect the clearance of Aβ(1–42). Neuronal Aβ levels were strikingly different after 48 h of treatment with the two peptides; relative levels of Aβ3pE-42 were 4.5-fold higher than cells treated with Aβ(1–42) (Fig. 4A). A trend toward higher mean levels of residual Aβ was observed when Aβ(1–42) solutions were supplemented with either 50 or 25%
(mol/mol) Aβ3pE-42; however, this effect was not observed when Aβ3pE-42 represented only 5% of the total Aβ concentration (Fig. 4A).

From experiments utilizing sequential extraction of cells following Aβ exposure, both the Aβ(1–42) and Aβ3pE-42 peptides were predominantly found in membrane fractions, with relatively little Aβ in the soluble (TBS) phase observed qualitatively by Western blot (Fig. 4B). Aβ in cell extracts resolved primarily as monomers on SDS-PAGE; however, bands corresponding to low-mass oligomers (~8–20 kDa) were observed in longer blot exposures.

**Toxicity of Pyroglutamate-Aβ Is Associated with Membrane Damage but Not Cytosolic ROS Production**—The capacities of each peptide to produce elevations in cytoplasmic and membrane ROS were studied using primary cortical mouse neurons. The fluorescent ROS probe DCF was used to measure cytoplasmic ROS flux over a 1-h treatment with freshly prepared Aβ peptides. Aβ(1–42) induced an approximate 10-fold increase in DCF fluorescence compared with control cells, whereas Aβ(1–40), Aβ3pE-40, or Aβ3pE-42 treatments were not found to be statistically different from controls (Fig. 5, A and B).

Minor quantities of Aβ3pE-42 were found to significantly disrupt the neuronal ROS-inducing capacity of Aβ(1–42); the ROS flux induced by solutions of Aβ(1–42) was diminished by 47% when Aβ3pE-42 was present at 5% (mol/mol) of total Aβ (Fig. 5B).

To assess Aβ induction of ROS at neuronal membranes, we directly measured lipid peroxidation in living cortical mouse neurons using the fluorescent probe DPPP over a 4-h treatment. In direct contrast to the DCF assays, Aβ(1–42) (10 μM) did not increase neuronal DPPP fluorescence, whereas cells treated with Aβ3pE-42 (10 μM) demonstrated a statistically sig-
Aβ in the human brain is represented by a heterogeneous and dynamic mixture of isoforms, with significant compositional variation between individuals (1–3, 5, 51). The predominance of pE-Aβ in the central core of plaques suggests an early involvement in amyloid deposition in the AD brain (52), whereas correlation between pE-Aβ and a decline in Mini Mental State Examination scores implicates pE-Aβ cytotoxicity in AD neurodegeneration (53, 54). In parallel, markers of oxidative stress are among the earliest detectable pathological changes in transgenic AD mouse models (55) and the human AD brain (36, 56, 57), with numerous lines of evidence implicating Aβ as a central contributor to oxidative stress in AD (58, 59).

Our data indicate that full-length Aβ and pE-Aβ exert different oxidative insults upon neurons, representing different mechanisms of neurotoxicity. Aβ(1–42) was found to significantly increase cytosolic ROS levels, whereas Aβ3pE-42 induced lipid peroxidation in the absence of cytosolic ROS flux. Additionally, we found that the neuronal membrane damage caused by Aβ3pE-42 results in a functional loss of plasma membrane integrity. These findings are consistent with recent publications demonstrating the capacity for Aβ3pE-42 to form membrane-disrupting pores in synthetic lipid bilayers (22, 23). Similarly, previous studies have shown that pE-Aβ oligomers disrupt lysosome membrane integrity in cultured neurons (50) and cause lactate dehydrogenase leakage from cultured astrocytes (49).
The earliest detectable effect of Aβ3pE-42 on neuronal homeostasis that we observed was the capacity to cause rapid Ca²⁺ influx, an effect that was partially ameliorated by pre-treatment of neurons with the NMDAR antagonist MK-801. The Ca²⁺ flux induced by Aβ3pE-42 appears to be separate from its capacity to undergo copper-redox cycling as the effect persisted when media were depleted of row 1 transition metals. Changes in cellular Ca²⁺ homeostasis induced by Aβ have previously been implicated in Aβ toxicity and are thought to occur via multiple mechanisms such as pore formation and NMDAR activation (60, 61). Importantly, however, the aggregation state of the Aβ preparation significantly affects the capacity to induce Ca²⁺ flux; monomeric (freshly prepared) and fibrillar Aβ(1–42) is not found to induce Ca²⁺ flux in SH-SY5Y neuroblastomas, whereas oligomeric preparations agonize NMDAR and trigger rapid Ca²⁺ flux (61, 62). Similarly, we found freshly prepared Aβ(1–42) to cause only modest elevation in cortical neuron Ca²⁺ levels above controls, which is contrasted by the much larger Ca²⁺ flux induced by Aβ3pE-42. Collectively, these data indicate that the unique neurotoxicity of pE-Aβ peptides is exerted through multiple interactions at the cell surface, including activation of NMDAR pathways, subsequently followed by peroxidation of membrane lipids and a loss of membrane integrity.

The potential for amino-truncated Aβ and pE-Aβ to alter the oligomerization and toxicity of full-length Aβ has been an area of recent debate and speculation. Aβ3pE-42 has a demonstrated capacity to dramatically enhance the aggregation of full-length Aβ (14), as does Aβ3–42 (41). Aβ3pE-42 has further been reported to enhance the toxicity of Aβ(1–42) in a prion-like seeding mechanism in cortical neuron cultures (24). Due to the strong membrane association of Aβ3pE-42, we initially predicted Aβ3pE-42 seeds to shift the ROS generation of Aβ(1–42) to a lipid compartment, thereby increasing Aβ(1–42)-induced lipoperoxidation; however, Aβ3pE-42 was not found to increase Aβ(1–42) ROS production in either the cytosolic or membrane fractions. In contrast, the capacity for Aβ3pE-42 seeds to significantly reduce Aβ(1–42) ROS production indicates that Aβ3pE-42 does not enhance Aβ(1–42) toxicity via ROS production, yet it suggests significant interaction between the peptides in neuronal cultures. This effect may be a result of accelerated Aβ(1–42) aggregation in the presence of Aβ3pE-42, as previous studies have found that Aβ-ROS production decreases with aggregation (32, 63). In the study by Nussbaum et al. (24), trace quantities of Aβ3pE-42 were found to enhance the toxicity of Aβ(1–42) when co-aggregated prior to cell treatment; however, when Aβ3pE-42 was seeded into Aβ(1–42) solutions immediately before applying to cells (as in our experiments), the toxicity of the seeded mixture was identical to the individual Aβ(1–42) treatment. It is therefore likely that Aβ aggregates possess different cytotoxic properties depending on both the composition of the Aβ mixture and the
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FIGURE 6. Measurement of Ca\(^{2+}\) flux in cortical mouse neurons using the Fluo-4 Ca\(^{2+}\) sensor. A, representative kinetics during the acute exposure of neurons to freshly prepared Aβ (10 μM). B, levels of Ca\(^{2+}\) flux induced by exposure to Aβ(1–42) and Aβ3pE-42 (10 μM each peptide) relative to glutamate-treated cells (Glu; 1 μM). Neurons were additionally pre-treated for 15 min with or without the NMDAR antagonist MK-801 (1 mM) to assess the contribution of NMDAR activation in Aβ3pE-42-induced Ca\(^{2+}\) flux. C, effect of transition metal depletion on Aβ-induced Ca\(^{2+}\) flux in neurons pre-treated for 1 h with the chelator Diamsar. D, separate Ca\(^{2+}\) flux assays were performed in HBSS buffer devoid of Ca\(^{2+}\) and Mg\(^{2+}\) to determine the source of Ca\(^{2+}\) moving into the cytosol. Each treatment was tested in a minimum of four replicate wells per assay and the experiments repeated at a minimum of three times over separate days. Data are represented by box and whiskers (Tukey method); statistical significance is shown above bars as relative to the vehicle treatment (PBS) or between treatments as indicated. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ns, not significant, p > 0.05.

timing of the Aβ isoform interactions. The possibility also exists that Aβ3pE-42 seeded mixtures cause oxidative damage that escape detection by the DCF and DPPP fluorescence ROS probes or possess different redox cycling capacities in other cerebral cell types (e.g. astrocytes and microglia). Other prevalent amino-truncated and pE-Aβ peptides found in AD brain tissues, such as Aβ(3–42), Aβ(4–42), and Aβ11pE-42 (2, 52), require further investigation as they also demonstrate enhanced amyloid-seeding capacity and could potentially alter Aβ-ROS dynamics.

AFM and SEC analysis revealed that Aβ(1–42) and Aβ3pE-42 oligomers differed not only in the rate of formation but also in size and structure. Previous studies have similarly reported differences between Aβ(1–42) and Aβ3pE-42 in the profile of oligomers and fibril ultrastructures formed in aqueous buffers (18, 30). Lee et al. (22) report that Aβ3pE-42 forms larger oligomers in synthetic lipid membranes than Aβ(1–42) and with faster kinetics of assembly, although it remains to be determined how this relates to the relative level of toxicity. Aβ3pE-42 neurotoxicity has also been demonstrated with a broad range of size fractions (monomers to >100 kDa), whereas the toxicity of Aβ(1–42) fractions was isolated to oligomers with an observed mass larger than 14 kDa (25). This is consistent with our SEC findings demonstrating the relative stability of low-mass Aβ(1–42) species (<14 kDa) in aqueous solution and the observation that freshly prepared Aβ(1–42) is less neurotoxic than Aβ3pE-42. The metastable nature of Aβ peptides, however, presents many technical challenges in delineating “toxic” fractions from “benign” fractions as purification processes undoubtedly alter oligomerization kinetics. Likewise, Aβ peptides undergo significant structural changes in extended cell culture incubations; hence, it is pertinent to correlate toxicity markers with time-matched biophysical characterizations.

The Aβ3pE-40 and Aβ3pE-42 peptides have been found to resist proteolysis in astrocyte cultures (49) and accumulate in the lysosomes of astrocytes in cell culture and the AD temporal cortex (50). Consistent with these reports, we observed Aβ3pE-42 to resist clearance in neuronal cultures, remaining at significantly higher levels than Aβ(1–42) over extended incubations. Aβ3pE-42 was, however, not found to prevent the clearance of Aβ(1–42) from neural cultures when present at minor quantities (5% mol/mol), suggesting that Aβ3pE-42 does not transfer protease resistance to Aβ(1–42) when applied to cells as fresh preparations. This does not exclude the possibility that pE-Aβ may affect the clearance of full-length Aβ when the peptides are aggregated, which will require further investigation. The capacity for Aβ3pE-42 to resist proteolytic degradation in both neurons and astrocytes is highly relevant given that pE-Aβ peptides are found in the cores of amyloid plaques in the AD brain (52), suggesting that pE-Aβ peptides, once formed, are long-lived neurotoxins.
Dityrosine is another post-translational protein modification that confers resistance to cellular catabolism. Total dityrosine levels are elevated in the AD hippocampus, neocortex, and ventricular cerebrospinal fluid compared with cognitively healthy individuals (64), yet the contribution of Aβ to cerebral dityrosine formation is not well understood. Dityrosine cross-linked Aβ fibrils are resistant to formic acid digestion, and sections of AD brains display intense dityrosine immunoreactivity within plaques (39). The pE-Aβ isoforms demonstrated increased efficiency for dityrosine oligomer formation compared with full-length Aβ. This observation can likely be attributed to increased hydrophobicity and the propensity to oligomerize; dityrosine formation is dependent on both the production of Aβ-tyrosyl radicals and the close proximity of Aβ molecules to allow tyrosine-tyrosine coupling (33). It is reasonable to speculate that the capacity for amyloid plaques to resist solubilization and clearance is due to the contribution of both the pE and dityrosine modifications to Aβ, which may account for the abundance of pE-Aβ in plaque cores (52).

Our data demonstrate clear differences in the neurotoxic mechanisms of pE-Aβ and full-length Aβ. The toxicity of pE-Aβ has recently been highlighted in mouse models overexpressing the soluble isoform of QC, demonstrating exacerbated neurodegeneration and behavioral deficits when crossed with mouse lines overexpressing Aβ or amyloid precursor protein transgenics (65, 66). The specific targeting of pE-Aβ via inhibition of QC-catalyzed pyroglutamate synthesis has demonstrated promising results; transgenic AD mice treated with a QC inhibitor show reduced plaque load, reduced gliosis, and an improvement in context memory and spatial learning (67). QC knock-out does not completely inhibit pE-Aβ formation in transgenic AD mouse models, however (68), suggesting that multiple pathways of pE-Aβ formation may exist. Antibodies currently under development as passive vaccine therapies for AD show significant differences in their capacity to bind amino-truncated Aβ species (69) and thus may fail to remove pE-Aβ and its precursors (Aβ3–40/42 and Aβ11–40/42). Individuals with AD may therefore require different therapeutic interventions to target distinct Aβ isoforms and their specific mechanisms of toxicity. Aβ ROS production, oligomerization, and dityrosine formation are potential therapeutic targets for AD; metal-protein attenuating compounds that inhibit these reactions are found to effect a marked decrease in amyloid deposition and improvement in cognitive deficits in transgenic AD mice (70, 71). Our observations indicate that the separate pathways of oxidative damage and neurotoxicity exerted by Aβ3pE-42 potentially have a unique contribution to AD pathology. These findings elicit further consideration of pE-Aβ peptides as targets in the pursuit of biomarkers and disease-modifying therapeutics for AD.

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**References**


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