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Original Article

Regulation of intracellular calcium in cortical neurons transgenic for human Aβ40 and Aβ42 following nutritive challenge

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Abstract: The pathogenesis of Alzheimer's Disease (AD) is not fully understood. Amyloid plaques could be causally linked to neuronal loss in AD. Two proteolytic products of the Amyloid Precursor Protein (APP), Amyloid β40 (Aβ40) and Amyloid β42 (Aβ42), are considered to be critical in the neurodegeneration seen in AD. However, in transgenic mice that overexpress human Aβ40 or Aβ42, it was shown that Aβ42 was much more amyloidogenic than Aβ40. In contrast to this observation, we have found that cultured cortical neurons from mice transgenic for human Aβ40 and for Aβ42 are both and statistically equally vulnerable to nutritive challenge induced by trophic factor withdrawal (TFW). Aberrant regulation of $InsP_3R$ (Inositol triphosphate receptor)-mediated calcium release has been implicated in neuronal cell death. It is however not clear whether this pathway plays a critical role in cortical neurons transgenic for different species of human Aβ. We now report that Aβ40 and Aβ42 equally exacerbated intracellular calcium response to TFW in cortical neurons following TFW. When bradykinin (BK), a potent stimulant of $InsP_3R$-mediated calcium release from ER, was applied to these cells, wild-type (WT) neurons exhibited a steep rise in $[Ca^{2+}]_i$ but this was not observed in either Aβ transgenic type. Similarly, when 1 μM Xestopongin C (XeC), a specific blocker of $InsP_3R$, was applied to these neurons, WT cells showed a significant attenuation of increase in $[Ca^{2+}]_i$ following TFW, while elevation in $[Ca^{2+}]_i$ induced by TFW remained largely unchanged in Aβ40 and Aβ42 cells. Finally, when we treated these cells with a Ca^{2+} chelator (BAPTA; 10 μM), all three cell types had a marked attenuation of $[Ca^{2+}]_i$. These findings indicate that the exacerbated calcium dysregulation following TFW in Aβ transgenic neurons are likely to be mediated by calcium channels other than ER InsP3R receptors. Overall, our results also suggest that a highly amyloidogenic Abeta species, such as Aβ42, might not necessarily be significantly more neurotoxic than a less or non-amyloidogenic Abeta species, such as Aβ40.

Key words: Amyloid β-peptide, intracellular calcium, $InsP_3R$, endoplasmic reticulum, transgenic mice

Introduction

AD is a progressive neurological disorder characterized by memory loss, personality change, global cognitive decline and a variety of other functional impairments. Structural changes in the brain in AD are consistent with neuronal loss in those cortical areas that are critical for cognition, intellectual operations, personality and memory. There are two hallmark pathological characteristics of AD, amyloid plaques and neurofibrillary tangles. Plaques are extraneuronal deposits of amyloid β peptide while tangles are intra-axonal cytoskeletal anomalies comprising hyperphosphorylated tau protein. The principal pathogenetic hypothesis of AD, the amyloid cascade theory, posits that the proteolytic cleavage of Aβ42 from APP is increased in AD. Insoluble forms of Aβ42 are released from neurons into the extracellular space where the peptides deposits as plaques [1-4]. It is not clear how an increase in Aβ42 cleavage or plaque deposition contributes to neuronal loss that is ultimately responsible for the clinical phenotype of AD.

Numerous studies have shown that disordered Ca^{2+} homeostasis plays a role in AD pathology [5]. The balance between $[Ca^{2+}]_i$ and ER Ca^{2+}

content is mediated through two distinct processes. On the one hand, Ca^{2+} is taken up by the ER against a gradient by Sarcoplasmic/ Endoplasmic Reticulum ATP-ases (SERCA) and released from this organelle via RyR and $InsP_3R$ [6]. Several authors have reported abnormal ER Ca^{2+} release from $InsP_3R$ pathways [7-9]. The ER is critical in the regulation of $[Ca^{2+}]_i$, in the folding and processing of many proteins and in the activation of cell death pathways [10]. It has been reported that when Ca^{2+} release from the ER is disturbed, extra-cellular accumulation of misfolded proteins can occur and ER-stress-induced apoptosis pathways can be triggered [11-13]. When cells are stressed (such as might occur under conditions of nutritive challenge), the ER and other organelles are also perturbed. Under these conditions, the ER chaperone GRP 78 is induced [14], Caspase-12 (residing on the cytoplasmic side of the ER), is activated [15] and this further activates Caspases-9 and -3 which commit the cell to apoptosis [16]. It has also been demonstrated that Caspase-12 mice are resistant to ER stress and to Aβ induced cell death [15]. In vitro studies have also shown that ER Ca^{2+} dyshomeostasis are synergistically related [11]. ER function itself is sensitive to the presence of reactive oxygen species (ROS) [17-19]. In addition, when Ca^{2+} is released from the ER is increased, the ion is increasingly taken up by mitochondria. The mitochondrial respiratory then is induced to generated further ROS [20]. Many studies have also demonstrated that oxidative stress is critical in mediating the neurotoxic properties of Aβ species [21, 22]. Furthermore, cells expressing AD-associated Presenilin-1 mutations have also been shown be susceptible to ER stress [23, 24]. Various laboratories have also reported that ER Ca^{2+}-release channels (RyR and InsP3R) are involved in transduction of the apoptosis signal pathway [25-27]. Apoptosis induction has also been observed in a large volume of published data in response to diverse signals [28]. Ferreiro et al have also shown that Aβ25-35 and Aβ40 both induce an increase in $[Ca^{2+}]_i$ in neurons after a 24-hour incubation with these peptides [11, 21].

In 2005, McGowan and colleagues published the creation of a new transgenic model of AD. In fact, McGowan et al generated two related strains of mice, one overexpressing human Aβ40 (BRI-Aβ40) and the other Aβ42 (BRI-Aβ42), against the same WT background and without attendant upregulation of APP [29]. In 2008, our laboratory reported that cortical neurons cultured from new born BRI-Aβ40 and BRI-Aβ42 pups both showed greater vulnerability to trophic factor withdrawal (TFW)-induced apoptosis. Under these conditions both transgenic neurons had elevated oxidative stress, exacerbated mitochondrial dysfunction, upregulated apoptotic signaling, and increased DNA fragmentation and cell death compare to WT controls [30].

We now report that dysregulation of intracellular calcium homeostasis induced by nutritive stress was exacerbated in cortical neurons transgenic for Aβ42 or Aβ 40. However, enhanced calcium release from ER via $InsP_3R$ did not seem to play a critical role in this model.

Materials and methods

Mice

Experiments were performed on primary cortical neurons derived from one-day-old pups from a breeding colony of BRI-Aß40 (designated Aß40), BRI-Aß42 (designated Aß42) and wild-type (WT) mice. These transgenic mice were generated and maintained on a B6C3 background. The colony was established at the Comparative Medicine Animal Facility of the University of Oklahoma Health Sciences Center (OUHSC) from a breeding group provided by Dr. Eileen McGowan of the Mayo Clinic College of Medicine, Jacksonville, FL. For details of derivation technique and related information, refer to McGowan et al Neuron July 2005 [29]. All experimental procedures and animal use were approved by the Institutional Animal Care and Use Committee of OUHSC.

Primary neuronal cultures

Dissociated primary cultures of cortical neurons were prepared from postnatal day 1 mouse pups (Aβ40, Aβ42 and WT) using methods described in our previous studies [31]. Briefly, cortex were removed and incubated for 15min in Ca^{2+} and Mg^{2+} free Hank's balanced Saline solution (Invitrogen) containing 0.2% papain. Cells were dissociated by trituration and plated into polyethyleneimine-coated glass-bottom culture dishes containing Minimum Essential Medium

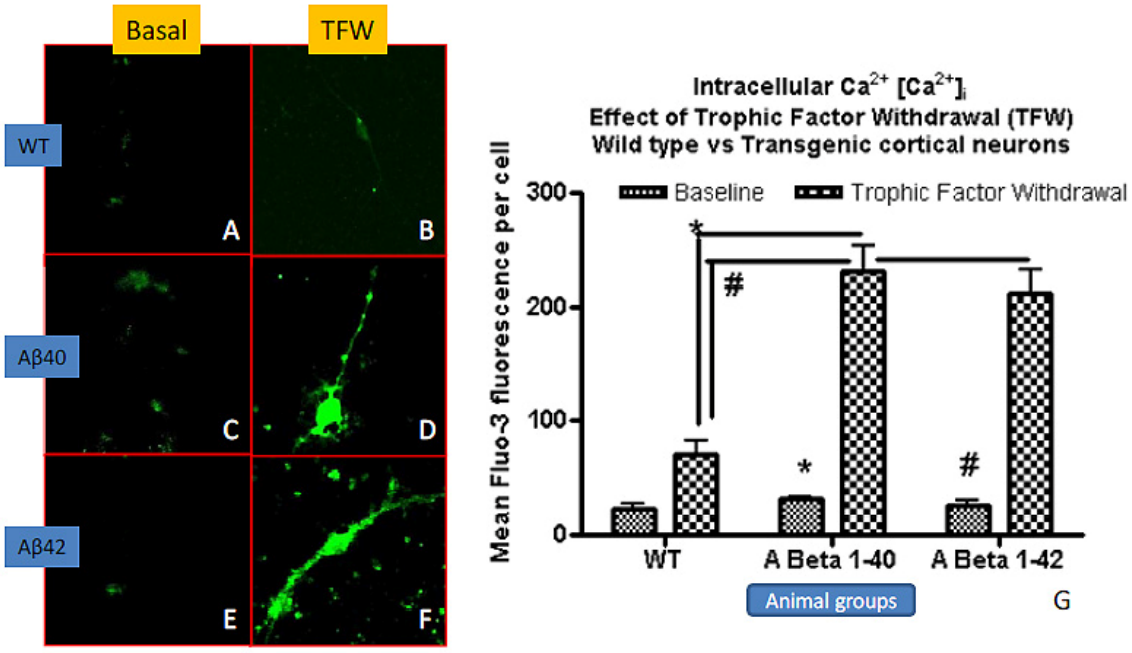

Figure 1: Calcium response to trophic factor withdrawal (TFW) was exacerbated in cortical neurons transgenic for both species of Amyloid β peptide. The cell permeant, Ca^{2+}-specific probe, Fluo-3 was used to capture confocal images of 1-week old cortical neuronal cultures. Samples were excited at 488 nm with a Ar/Kr laser. Emissions were recorded with filters chosen for 510 nm. TFW was achieved with incubations in glucose-free medium. PANEL A: Wild type (WT) neurons under basal conditions. PANEL B: WT neurons after 24 hours of TFW. PANEL C: Amyloid beta 1-40 transgenic neurons (Aβ40) under basal conditions. PANEL D: Aβ40 neurons after 24 hours TFW. PANEL E: Amyloid β 1-42 (Aβ42) neurons under basal conditions. PANEL F: Aβ42 neurons after 24 hours TFW. PANEL G: Bar plot of mean Fura-3 fluorescence intensity per cell (in semi-quntitative arbitrary values ±SE). WT Ctrl: corresponds to panel A; WT TFW: Panel B; Aβ40 Ctrl: Panel C; Aβ40 TFW: Panel D; Aβ42 Ctrl: Panel E; Aβ42 TFW: Panel F. One-way ANOVA with a Tukey post-hoc analysis was performed for statistical testing: * and # indicates statistical significance at p values of <0.05. Statistical significance was reached between Aβ40 TFW and WT TFW, Aβ40 TFW and Aβ40 Ctrl, Aβ42 TFW and WT TFW and Aβ42 TFW and Aβ42 Ctrl.

with Earle's salts supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 1mM pyruvate, 20mM KCL, 10mM sodium bicarbonate and 1mM Hepes pH7.2. Following cell attachment (3-6h post-plating), the culture medium was replaced with growth medium which is Neurobasal medium with B27 supplements (Invitrogen). Plated cells were allowed to grow for 7 days in 35 mm glass-bottom culture dishes before they were subjected to TFW conditions and/or other treatments. TFW was achieved with incubations in glucose-free medium, Locke's solution without glucose.

$[Ca^{2+}]_i$ Imaging by confocal laser scanning microscopy

For semi-quantitative [Ca2+]I analysis, culture dishes were loaded with 5 μM Fluo-3 AM (Ca^{2+}-specific, fluorescent probe; ANASPEC; Fremont, CA 94555) for 30 minutes in a cell culture incubator [32] before being examined using a Carl Zeiss LSM-510 META (Carl Zeiss Microimaging; Thornwood, NY 10594) laser scanning confocal microscope. Samples were excited with an Argon/Krypton laser tuned to 488 nm and a detector set to 510 nm. Images were stored as jpeg images on a PC for off-line analysis. Image analysis was performed with Image J software (public domain image analysis software by NIH image; Research Services Branch, National Institute of Mental Health, National Institutes of Health). Mean image intensity per cell was measured as arbitrary units of Fluo-3 intensity subtracted from background in each analyzed image and

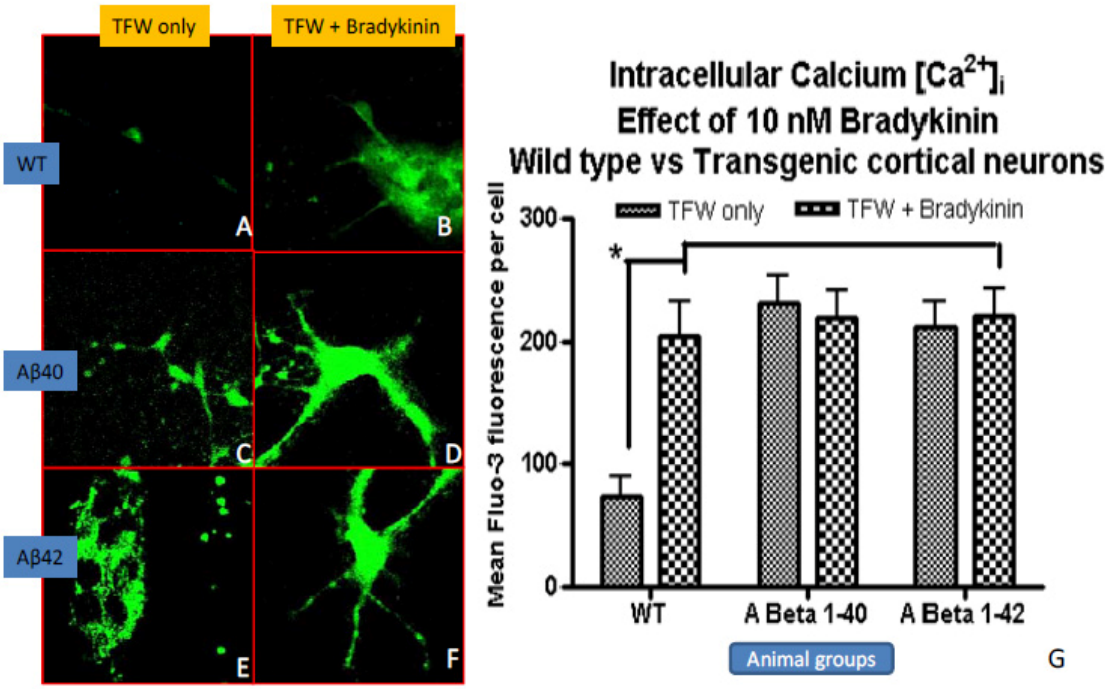

Figure 2: Stimulation of $InsP_3R$-mediated calcium release with bradykinin does not alter calcium response to TFW in cortical neurons transgenic for Aβ40 or Aβ42. Effect of 1-hour pretreatment with 10nM Bradykinin on intraneuronal Ca^{2+} ($[Ca^{2+}]_i$. The cell permeant, Ca^{2+}-specific probe, Fluo-3 was used to capture confocal images of 1-week old cortical neuronal cultures. Samples were excited at 488 nm with a Ar/Kr laser. Emissions were recorded with filters chosen for 510 nm. TFW was achieved with 24-hour incubations in glucose-free medium. PANEL A: Wild type (WT) neurons under TFW only conditions. PANEL B: WT neurons after 24 hours of TFW with 10nM Bradykinin. PANEL C: Amyloid β 1-40 transgenic neurons (Aβ40) under TFW conditions. PANEL D: Aβ40 neurons after 24 hours TFW with 10nM Bradykinin. PANEL E: Amyloid β 1-42 (Aβ42) neurons under TFW conditions. PANEL F: Aβ42 neurons after 24 hours TFW with Bradykinin. PANEL G: Bar plot of mean Fura-3 fluorescence intensity per cell (in semi-quntitative arbitrary values ±SE). WT (TFW): corresponds to panel A; WT (TFW+Brady.): Panel B; Aβ40 (TFW): Panel C; Aβ40 (TFW+Brady.): Panel D; Aβ42 (TFW): Panel E; Aβ42 (TFW+Brady.): Panel F. one-way ANOVA with Tukey post-hoc analysis was performed for statistical testing: * indicates statistical significance.

plotted using commercial plotting software (GraphPad Prism 4; GraphPad Prism Software, Inc., La Jolla, CA 92037).

Drugs and Chemicals

Xestospongin-C (XeC) was purchased from A.G. Scientific, San Diego CA 92121. Bradykinin (BK) and 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were purchased from Tocris Bioscience, Ellisville MO 63021. Culture dishes were loaded with 5 μM Fluo-3 AM probe [5 μM; [32]] for 30 minutes after treatment with one of these compounds. Treatment with XeC was for 45 minutes at a concentration of 1 μM [33], BK for 1 hour at 10 nM [34] and BAPTA for 45 minutes at a concentration of 10 μM [35].

Statistical analysis

Statistical differences between experimental groups were determined by performing one-way ANOVA followed by post-hoc Tukey's test for multiple comparisons against a control group. Data are reported as mean ± SEM fluorescence/cell in arbitrary units measured against background fluorescent intensity. Analysis was performed with 3 degrees of freedom (DF) and a 95% confidence interval (CI). Results were considered significant at a p value <0.05.

Results

Dysregulation of Intracellular calcium homeostasis following TFW was exacerbated in neurons transgenic for Abeta 42 or Abeta 40

When basal $[Ca^{2+}]_i$ was measured in WT, Aβ40 and Aβ42 cultured cortical neurons (n=12 animals per group; on average 4-6 neurons per microscopic field and 2-3 fields were assessed per animal), no differences were observed. However, following 14 hours of trophic factor withdrawal (TFW), $[Ca^{2+}]_i$ was observed to increase in all three neuronal cultures, with Aβ40 and Aβ42 transgenic cells showing dramatically higher increases than WT cells (WT after TFW: mean 70.25 ± 12.00 SE; Aβ40 after TFW: 231.77 ± 21.56 SE; Aβ42: 211.00 ± 21.65 SE). The increases in intracellular calcium levels in Aβ40 and Aβ42 transgenic neurons are not significantly different, indicating that Aβ40 and Aβ42 exacerbate intracellular calcium homeostasis to a similar degree following TFW. These data are consistent with our cell death data reported previously, and are summarized in **Figure 1**.

Stimulation of InsP3R-mediated calcium release with bradykinin failed to alter calcium response to TFW in cortical neurons transgenic for Aβ40 or Aβ42

To start examining if exacerbated calcium response to TFW in Aβ transgenic neurons was

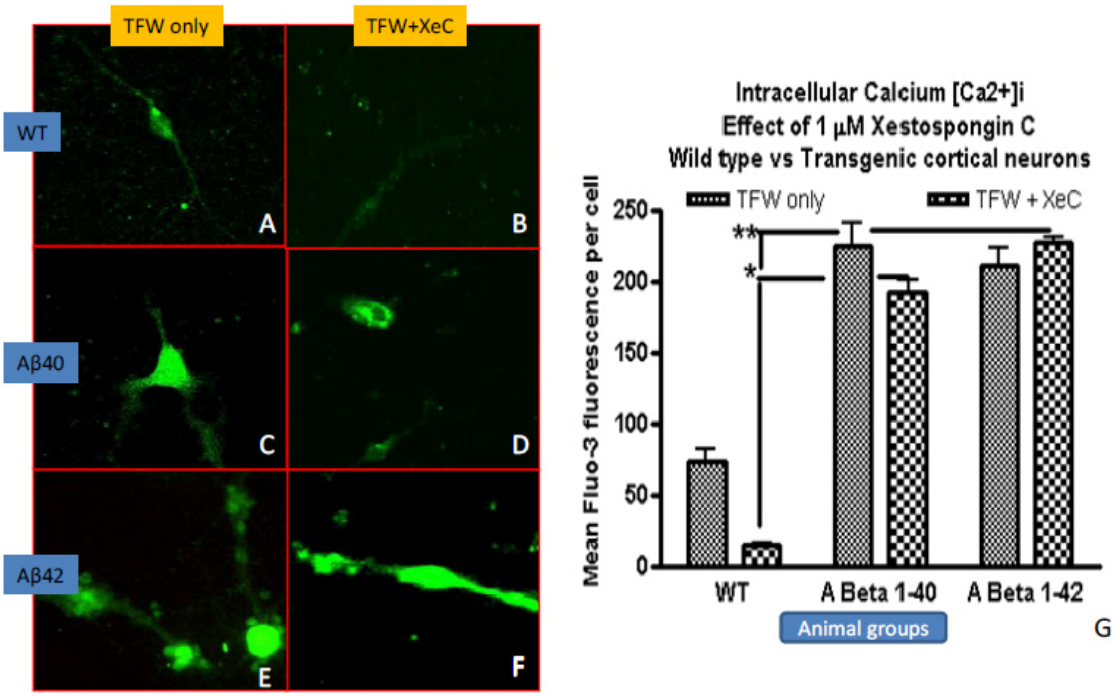

Figure 3: Blockade of $InsP_3R$-mediated calcium release from ER has no significant effects on calcium response to TFW in cortical neurons transgenic for Aβ40 or Aβ42. The cell permeant, Ca^{2+}-specific probe, Fluo-3 was used to capture confocal images of 1-week old cortical neuronal cultures. Samples were excited at 488 nm with a Ar/Kr laser. Emissions were recorded with filters chosen for 510 nm. TFW was achieved with incubations in glucose-free medium. PANEL A: Wild type (WT) neurons under TFW only conditions. PANEL B: WT neurons after 24 hours of TFW with 1 µM XeC. PANEL C: Amyloid β 1-40 transgenic neurons (Aβ40) under TFW conditions. PANEL D: Aβ40 neurons after 24 hours TFW with 1 µM XeC. PANEL E: Amyloid β 1-42 (Aβ42) neurons under TFW conditions. PANEL F: Aβ42 neurons after 24 hours TFW with 45-minute incubation with 1 µM XeC. PANEL G: Bar plot of mean Fura-3 fluorescence intensity per cell (in semi-quntitative arbitrary values ±SE). WT (TFW): corresponds to panel A; WT (TFW+XeC.): Panel B; Aβ40 (TFW): Panel C; Aβ40 (TFW+XeC): Panel D; Aβ42 (TFW): Panel E; Aβ42 (TFW+XeC): Panel F. One-way ANOVA with a Tukey post-hoc analysis was performed for statistical testing: * and ** indicate statistical significance at p values of <0.05. Statistical significance was reached between WT (TFW) and Aβ40TFW, Aβ40TFW+XeC, Aβ42TFW and Aβ42TFW+XeC, as well as WT TFW+XeC and Aβ40 TFW, Aβ40 TFW+XeC, Aβ42TFW and Aβ42 TFW+XeC.

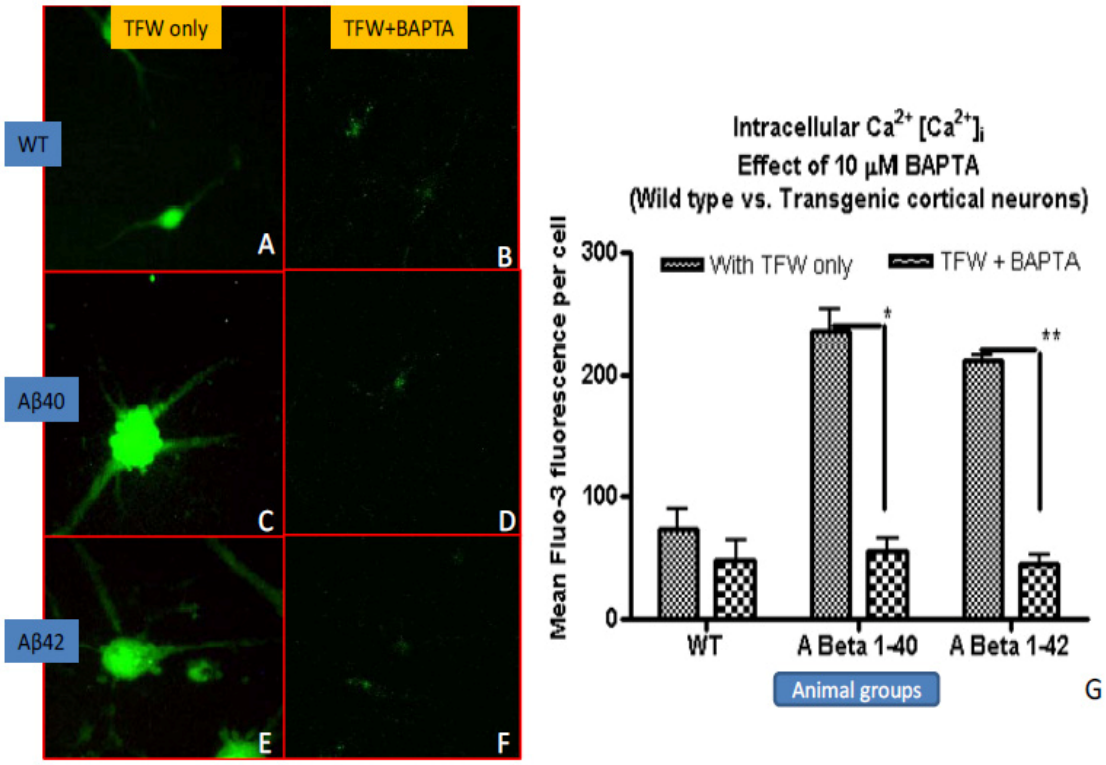

Figure 4: Chelation of intracellular free Ca^{2+} in WT, Aβ40 and Aβ42 expressing neurons. The cell permeant, Ca^{2+}-specific probe, Fura-3 was used to capture confocal images of 1-week old cortical neuronal cultures. Samples were excited at 488 nm with a Ar/Kr laser. Emissions were recorded with filters chosen for 510 nm. TFW was achieved with incubations in glucose-free medium. PANEL A: Wild type (WT) neurons under TFW only conditions. PANEL B: WT neurons after 24 hours of TFW and 45 minute incubation with 10 µM BAPTA. PANEL C: Amyloid β 1-40 transgenic neurons (Aβ40) under TFW conditions. PANEL D: Aβ40 neurons after 24 hours TFW and 10 µM BAPTA. PANEL E: Amyloid β 1-42 (Aβ42) neurons under TFW conditions. PANEL F: Aβ42 neurons after 24 hours TFW with 10 µM BAPTA. PANEL G: Bar plot of mean Fura-3 fluorescence intensity per cell (in semi-quntitative arbitrary values ±SE). WT +TFW: corresponds to panel A; WT +TFW+BAPTA: Panel B; AB40+TFW: Panel C; AB40+TFW+BAPTA: Panel D; Aβ42+ TFW: Panel E; Aβ42+TFW+BAPTA: Panel F. 1-way ANOVA with a Tukey post-hoc analysis was performed for statistical testing: * and ** indicate statistical significance at p values of <0.05. Statistical significance was reached between Aβ40+TFW and Aβ40+TFW+BAPTA; Aβ42+TFW and Aβ42+TFW+BAPTA.

mediated by an enhanced calcium release from ER via $InsP_3R$ receptors, we subjected cortical neurons to 14 hours of TFW, followed by 1 hour incubation with 10 nM of the potent inositol triphosphate receptor ($InsP_3R$) agonist bradykinin (BK). Intracellular calcium imaging was then performed by confocal microscopy (n=8 animals per group; on average 4-6 neurons per microscopic field and 2-3 fields were assessed per animal). $[Ca^{2+}]_i$ response to TFW was significantly enhanced by BK in WT neurons (rising from mean of 73.66 ± 16.27 SE to 204.00 ± 28.58 SE). However, the same BK treatment failed to alter calcium response to TFW in cortical neurons transgenic for either Aβ species. Calcium levels in Abeta40 expressing neurons actually decreased from mean of 231.75 ± 28.81 SE before BK to 218.75 ± 3.18 SE after BK, although this change is not statistically significant. In Aβ42 expressing neurons, BK induces only a very slight increase in $[Ca^{2+}]_i$ that was not statistically significant (TFW only levels: mean 211.00 ± 21.65 SE; TFW with BK: 220.66 ± 21.88 SE). The mechanisms underlying the muted response to BK in Aβ transgenic neurons are not clear, but it may indicate a depletion of ER calcium stores or a

desensitization of $InsP_3R$ to BK in these neurons following TFW. These data are summarized in **Figure 2**.

Blockade of $InsP_3R$-mediated calcium release from ER has no significant effects on $[Ca^{2+}]_i$ response to TFW in Aβ transgenic neurons

To further examine if $InsP_3R$-mediated calcium release from ER contributes significantly to the aberrant increase in $[Ca^{2+}]_i$ following TFW in Aβ transgenic neurons, we investigated the effect of XeC, a potent and specific antagonist of InsP3R. When 1 μM of XeC was applied to WT neurons and Aβ40 and Aβ42 transgenic cells (45 minute incubation in culture before TFW; n=6 animals per group; on average 4-6 neurons per microscopic field and 2-3 fields were assessed per animal), calcium response to TFW in WT cells was significantly attenuated ($p = <0.05$; TFW only mean: 73.66 ± 16.27 SE; TFW with XeC treatment: mean 15.66 ± 2.33SE). However, XeC did not induce statistically significant downward shifts in $[Ca^{2+}]_i$ in either Aβ40 or Aβ42 expressing neurons (Aβ40 TFW only: mean 224.7 ± 28.81 SE; TFW with XeC: mean 192.33 ± 15.30 SE; Aβ42 TFW only: mean 211.00 ± 21.65 SE; TFW with XeC: mean 227.33 ± 6.56 SE). These results suggest that the exacerbated calcium response to TFW in Aβ transgenic neurons is likely to be mediated by calcium channels other than ER $InsP_3R$ receptors. The data showing that the $[Ca^{2+}]_i$ response to TFW in neither transgenic cell types changed in a statistically significant manner following XeC are summarized in **Figure 3**.

$[Ca^{2+}]_i$ chelation with BAPTA significantly attenuates the calcium response to TFW in wild-type as well as Aβ transgenic neurons

When the highly specific, cell permeant Ca^{2+} chelator BAPTA was used to treat cultured WT, Aβ40 and Aβ42 expressing neurons, $[Ca^{2+}]_i$ response to TFW were dramatically attenuated (45 minute treatment with BAPTA at a concentration of 10 μM; n=8 animals per group; on average 4-6 neurons per microscopic field and 2-3 fields were assessed per animal) in all three cell types. Ca^{2+} levels dropped from 73.66 (mean ± 16.27 SE, TFW) to 47.75 (mean ± 23.54 SE, TFW+BAPTA) in WT; 236.33 (mean ± 17.18 SE, TFW) to 55.66 (mean ± 11.09 SE, TFW+BAPTA) in Aβ40 and 211.00 (mean ± 4.50 SE, TFW) to 44.50 (mean ± 5.50 SE, TFW+BAPTA) in Aβ42 neurons. These data are summarized in **Figure 4**.

Discussion

The data from this study demonstrate that: (1) Statically, Aβ40 and Aβ42 equally exacerbated intracellular calcium response to TFW in cortical neurons; (2) Stimulation of $InsP_3R$-mediated calcium release with bradykinin failed to alter calcium response to TFW, and blockade of $InsP_3R$-mediated calcium release from ER had no significant effects on $[Ca^{2+}]_i$ response to TFW in cortical neurons transgenic for Aβ40 or Aβ42. These results indicate that overexpression of Aβ40 or Aβ42 significantly alters calcium regulation following TFW, but an enhanced calcium release from ER mediated by $InsP_3R$ may not play a major role in this model.

This report represents research that is a mechanistic extension to previously reported findings from our laboratory [30]. In the previous work we found that overexpression of Aβ40 and Aβ42 almost equally increased neuronal susceptibility to oxidative stress, mitochondrial dysfunction and apoptotic cell death when the cellular environment becomes hostile under nutritive stress induced by TFW, which simulates the conditions of an aged Alzheimer's brain. Under these conditions, vascular supply, energy utilization, cellular metabolism and waste clearance by the brain are all known to be compromised. Several lines of evidence have implicated neuronal changes in $[Ca^{2+}]_i$ being critical in AD neurodegenration. For example, Ca^{2+} homeostasis is altered in fibroblasts isolated from AD patients [36], in cells bearing the human presenilin 1 (PS1) AD mutation [7], and evidence for abnormal ER Ca^{2+} release through the ER $InsP_3$ receptor [9]. The findings from the present study showing that Aβ40 and Aβ42 statistically equally exacerbated calcium response to TFW are consistent with our cell death data from the previous study, and confirms that dysregulation of intracellular calcium is critically associated with neuronal cell death induced by both Aβ40 and Aβ42. These results are in sharp contrast to the published data that demonstrated significant difference in amyloidogenic properties between the two Aβ species. Aβ42 was shown to be much more amyloidogenic than Aβ40 despite a very similar property in neurotoxicity demonstrated in our studies. These

observations are important because, contrary to traditional way of thinking, we show that a highly amyloidogenic Aβ species (such as Aβ42) might not necessarily be more neurotoxic than a non-amyloidogenic Aβ species (such as Aβ40).

Our data on $InsP_3R$ are also somewhat unexpected, given the documented role of calcium release from ER via these receptors in several models of neurodegeneration. Our findings of exacerbated $[Ca^{2+}]_i$ response in both Aβ transgenic neurons are strongly suggestive of a mechanism involving disturbed Ca^{2+} homeostasis when these cells are exposed to nutritional stress. However, the hypothesis that this increase might be mediated through ER Ca^{2+} release via $InsP_3R$ is not supported by our data where stimulation of $InsP_3R$ with BK and blockade of these receptors with XeC did not yield an appreciable impact on calcium response following TFW in transgenic cells even though WT neurons responded to these treatments in a predictable manner. The mechanisms underlying the muted response to BK in Aβ transgenic neurons are not clear, but it may indicate a depletion of ER calcium stores or a desensitization of $InsP_3R$ to BK in these neurons following TFW. A third possibility could be that in neurons transgenic for Aβ, Fluo-3 AM (the acetoxymethyl ester of Fluo-3) was already saturated with increased intracellular calcium following TFW to a point that stimulation with BK would not be able to increased the fluorescence further even when additional calcium was being released from ER. However, this scenario is highly unlikely for the following reasons: Based both on published literature as well as recommendations from the dye's manufacturer (ANASPEC; Fremont, CA 94555), we optimized the use of Fluo-3 AM by selecting recommended concentrations of the dye (5 μM), loading time (35 minutes) and incubation temperature (37°). Several lines of published evidence suggest that average basal level of intracellular free calcium in primary cortical neurons is about 80-90 nM, and that at this concentration of calcium, only about 15% of Fluo-3 is typically bound to calcium (Stephen Paddock et al., Confocal Microcopy Methods and Protocols, Humana Press, 1999). Saturation of Fluo-3 by calcium typically does not occur until free calcium is well over 3 μM, which would require an increase of over 30 times above the average resting calcium level in these neurons. The increase that we observed in Aβ transgenic neurons was ~3 folds over the baseline level, which was far below what would be expected for saturation of Fluo-3. The apparent lack of calcium response to XeC in Aβ transgenic neurons following TFW indicates that calcium dysregulation in these cells following TFW are likely to be mediated by calcium channels other than ER $InsP_3R$ receptors. The fact that we were able to significantly dampen the increase $[Ca^{2+}]_i$ with chelation of the free ion with BAPTA in all three types of neurons, coupled with the published observations that specific calcium channel blockers may confer neuroprotective actions, suggests that it is imperative to pursue cell survival strategies in AD models based on mechanisms that are critically involved in maintaining intracellular calcium homeostasis.

The precise calcium channels (or some other modality responsible for increased $[Ca^{2+}]_i$) that are directly involved in aberrant calcium response to TFW in Aβ transgenic neurons remain to be identified. Various specific calcium channels on the plasma membrane and many calcium release channels (other than $InsP_3R$) on the ER could be responsible for the exacerbated calcium dysregulation in Aβ expressing neurons observed in this study. The potential role of an aberrant calcium channel formed by Aβ itself is also intriguing since oligomeric forms of Aβ has been shown to have the ability to insert into the plasma membrane of neurons and act as aberrant, Ca^{2+}-specific channels.

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