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Enhanced caffeine-induced Ca^{2+} release in the 3xTg-AD mouse model of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is the most prevalent form of dementia among the elderly and is a complex disorder that involves altered proteolysis, oxidative stress and disruption of ion homeostasis. Animal models have proven useful in studying the impact of mutant AD-related genes on other cellular signaling pathways, such as Ca^{2+} signaling. Along these lines, disturbances of intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis are an early event in the pathogenesis of AD. Here, we have employed microfluorimetric measurements of $[Ca^{2+}]_i$ to investigate disturbances in Ca^{2+} homeostasis in primary cortical neurons from a triple transgenic mouse model of Alzheimer's disease (3xTg-AD). Application of caffeine to mutant presenilin-1 knock-in neurons ($PS1_{KI}$) and 3xTg-AD neurons evoked a peak rise of $[Ca^{2+}]_i$ that was significantly greater than those observed in non-transgenic neurons, although all groups had similar decay rates of their Ca^{2+} transient. This finding suggests that Ca^{2+} stores are greater in both $PS1_{KI}$ and 3xTg-AD neurons as calculated by the integral of the caffeine-induced Ca^{2+} transient signal. Western blot analysis failed to identify changes in the levels of several Ca^{2+} binding proteins (SERCA-2B, calbindin, calsenilin and calreticulin) implicated in the pathogenesis of AD. However, ryanodine receptor expression in both $PS1_{KI}$ and 3xTg-AD cortex was significantly increased. Our results suggest that the enhanced Ca^{2+} response to caffeine observed in both $PS1_{KI}$ and 3xTg-AD neurons may not be attributable to an alteration of endoplasmic reticulum store size, but to the increased steady-state levels of the ryanodine receptor.

Keywords: Alzheimer's, caffeine, calcium, neuron, presenilin, ryanodine.

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Alzheimer's disease (AD) is the most common form of age-related dementia among the elderly. A defining feature of AD is the appearance of diffuse and neuritic plaques composed of the amyloid beta (Aβ) peptide and neurofibrillary tangles comprising hyperphosphorylated tau protein. The mechanisms of neuronal degeneration in AD are not well understood, yet there is a growing consensus that cell death is at least in part due to dysregulated Ca^{2+} signaling (LaFerla 2002). More importantly, the alteration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) homeostasis occurs much earlier than many of the histopathological markers seen in the AD brain, suggesting that disturbances of $[Ca^{2+}]_i$ handling are an early pathogenic feature of AD (Chui *et al*. 1999).

The endoplasmic reticulum (ER) contains two main types of Ca^{2+} release channels, the inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine receptor (RyR). IP_3 stimulates Ca^{2+} release from the IP_3R, whereas Ca^{2+} ions stimulate the release of Ca^{2+} through the RyR. RyR-mediated Ca^{2+}-induced Ca^{2+} release (CICR) regulates many neuronal functions such as spike activity, synaptic plasticity, modulation of membrane excitability and gene expression (Zucker 1996; Krizaj *et al*. 1999; Rose and Konnerth 2001; Toescu *et al*. 2004). It is not surprising, therefore, to find repeated studies identifying disturbances of $[Ca^{2+}]_i$ handling in a host of pathologies, including AD and Huntington's disease (LaFerla 2002; Panov *et al*. 2002; Smith *et al*. 2002; Bezprozvanny and Hayden 2004). Several studies have focused on the contribution of mutant presenilin 1 (PS1), which accounts for the majority of early onset AD cases. Indeed, there are numerous reports identifying dysregulation

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Abbreviations used: Aβ, amyloid beta; AD, Alzheimer's disease; APP, amyloid precursor protein; $[Ca^{2+}]_i$, intracellular free Ca^{2+}concentration; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; MEM, minimum essential medium; NCX, Na^+/Ca^{2+} exchanger; PMCA, plasma membrane Ca^{2+} ATPase; PS1, presenilin 1; RyR, ryanodine receptor; 3xTg-AD, triple transgenic mouse model of Alzheimer's disease.

of Ca^{2+} signaling conferred by PS1 mutations as playing a causal role in the pathogenesis of early onset AD cases. For example, expression of mutant PS1 in both cultured cells and primary neurons has been shown to enhance Ca^{2+} transients (Leissring *et al*. 1999; Chan *et al*. 2000; Smith *et al*. 2002). In particular, PC12 cells and mouse cortical neurons expressing mutant forms of PS1 show an enhancement of cytosolic Ca^{2+} following exposure to the agonist, caffeine (Chan *et al*. 2000). It has also previously been demonstrated that liberation of Ca^{2+} from RyR-sensitive Ca^{2+} stores can modulate the processing of amyloid precursor protein (APP), resulting in an enhanced level of the Aβ peptide (Querfurth *et al*. 1997).

Transgenic mice have proven extremely useful for modeling various aspects of AD pathology. Recently, our laboratory developed a triple-transgenic model of Alzheimer's disease (3xTg-AD) by introducing two additional transgenes (APP_{swe} and tau_{P301L}) into the germline of the PS1 mutant knock-in mouse (Oddo *et al*. 2003). These transgenes are expressed under the transcriptional control of the Thy1.2 cassette, and develop an age-related and neuropathological phenotype that includes both plaque and tangle pathology. The human Swedish APP mutation is a double mutation located near the cleavage site of the β-secretase, resulting in enhanced β-secretase activity and an enhanced production of total Aβ (Cai *et al*. 1993). It is interesting to note that virtually every important derivative of APP, including the secreted Aβ peptide, the β-carboxy-terminal fragment (C99) and, most recently, the α-CTF (otherwise known as the APP intracellular domain) all modulate Ca^{2+} dynamics in some way (LaFerla 2002). The tau gene mutation associated with frontotemporal dementia with parkinsonism linked to chromosome 17 was used to facilitate the development of tau deposition in these mice, yet the effect of the tau mutation on $[Ca^{2+}]_i$ stores has not previously been investigated in primary neurons.

There is a growing body of evidence implicating defects in Ca^{2+} regulation as the early molecular defect in the pathogenesis of AD. In this study, we investigated whether Ca^{2+} homeostasis is disrupted in primary neurons isolated from the 3xTg-AD mice. Our findings indicate that 3xTg-AD neurons display enhanced Ca^{2+} release when challenged with caffeine. The mechanism underlying this effect appears to be due to higher levels of the RyR.

Materials and methods

Cortical cultures

Primary cortical neurons were prepared from embryonic day 15 (E15-18) NonTg and homozygous 3xTg-AD mice. Briefly, cerebral cortices were removed and incubated for 10 min in minimal essential medium (MEM) (Gibco, Grand Island, NY, USA) containing 0.125% trypsin. Trypsin digestion was halted by the addition of an equal volume of MEM containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). The tissue was then pelleted by centrifugation at 1800 *g* for 5 min and resuspended in 2 mL plating medium (MEM supplemented with glutamine, 10% horse serum and 10% FBS). The tissue was subsequently triturated 10 times with two fire-polished pipettes of narrowing bore size. After allowing larger pieces of tissue to settle for 5 min, 2×10^5 cells were plated down on poly D-lysine-coated coverslips. Following cell attachment (24 h after plating), the culture medium was replaced with neurobasal medium supplemented with B27 and L-glutamine. Every 4 days, half of the culture medium was replaced with fresh medium. Experiments were performed on 12- to 17-day-old cultures.

Microfluorimetric recordings

To measure cytosolic Ca^{2+}, glass coverslips onto which neurons had been grown were incubated in 2 mL control solution containing 4 μM fura-2AM for 30 min in the dark, followed by a 30 min de-esterification period at 21–24°C. Control solution was composed of (mM): 120 NaCl, 4 KCl, 0.2 $MgCl_2$, 15 glucose, 20 HEPES, 2.5 $CaCl_2$. Following the incubation period, fragments of coverslips were transferred to a recording chamber mounted on the stage of an inverted microscope and cells were continuously perfused under gravity at a rate of 1–2 mL/min. $[Ca^{2+}]_i$ was determined using the InCyt Im2 Ratio Imaging System (Intracellular Imaging Inc., Cincinnati, OH, USA) using fura-2AM excited alternatively at 340 and 380 nm.

For cytosolic Ca^{2+} measurements, several parameters were determined from the collected data. Changes in $[Ca^{2+}]_i$ were obtained by measuring the peak values and expressing them as a change in the Ca^{2+} concentration. The time course of the fluorescence signal decay was described by a single exponential equation, and the time constant (τ) was used as a measure of the rate of decline of $[Ca^{2+}]_i$. The size of caffeine-evoked stores are taken from the integral of the transient response. All results are expressed as means ± SE, together with sample traces, and statistical comparisons were made using unpaired Student's *t*-tests. For all experiments recorded, 3–6 cells in any one field were selected before the experiment was performed. At least five repeats of each experiment were performed.

Western blotting

Cortices from E15 transgenic and NonTg mice were homogenized in T-Per tissue protein extraction reagent (Perbio Science, Cramlington, UK) containing Complete Mini Protease Inhibitors (Roche Diagnostics, Pleasanton, CA, USA). The homogenized mixes were centrifuged at 4°C for 1 h at 100 000 *g*. The supernatant fluid was used for immunoblot analysis. Protein levels in the cell lysates were measured using the method of Bradford (Bradford 1976). Proteins (20 μg per lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 3–8% Tris-acetate for RyR or 10% bis-tris for all other proteins (Invitrogen, Carlsbad, CA, USA), under reducing conditions, and transferred for 2 h onto 0.2 μm nitrocellulose membranes. Membranes were blocked with 5% non-fat milk for 1 h at 20°C. Following overnight incubation at 4°C with secondary antibodies [(RyR, 1 : 500 (Abcam, Cambridge, MA, USA); calsenilin, 1 : 100 (Zymed, San Francisco, CA, USA); calreticulin, 1 : 1K (Chemicon, Temecula, CA, USA); calbindin, 1 : 5K (Chemicon) and SERCA-2B, 1 : 5K), blots were washed in Tween–Tris-buffered saline (TBS) for 20 min and incubated at 20°C

with horseradish peroxidase-conjugated secondary antibody. Bands were visualized using Supersignal West Femto (Pierce, Rockford, IL, USA) and CL-XPosure film (Pierce). Band intensities were measured using Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

Results

3xTg-AD mice express APP and tau transgenes during embryogenesis

The mouse Thy1.2 expression cassette has been demonstrated to drive expression predominantly, if not exclusively, to the central nervous system, particularly restricting expression to neurons (Caroni 1997). It has previously been reported that expression and activity of Thy1.2-driven transgenes is very low until postnatal day 6–12 (Caroni 1997). This developmental delay presents a potential drawback for studies, such as those focused on the impact on Ca^{2+} homeostasis, that involve the use of dissociated neuronal cultures. The 3xTg-AD mice harbor the M146V mutation knocked-in to the PS1 gene and overexpress human APP_{swe} and Tau_{P301L}, under the transcriptional control of the Thy1.2 expression cassette (Oddo *et al*. 2003). To determine whether the human APP and human tau proteins were expressed during late embryogenesis, and thereby to ascertain whether embryonic neurons could be studied, cortical brain regions were dissected from E15 3xTg-AD mice and protein extracts were analyzed by western blot. We showed a threefold increase in steady-state levels of both the APP and tau proteins in homozygous mice (Fig. 1). This finding is in contrast with adult homozygous 3xTg-AD mice, which showed a sixfold increase in steady-state levels of APP and tau (Oddo *et al*. 2003), suggesting that expression levels had not yet peaked. Nevertheless, these harvested neurons are still useful for studying Ca^{2+} signaling alterations as they express APP and tau.

Caffeine-induced Ca^{2+} release is enhanced in $PS1_{KI}$ and 3xTg-AD neurons

Methylxanthines such as caffeine lower the threshold for activation of the RyR promoting caffeine-induced Ca^{2+} release and have been widely used in the studies of sensory neurons (Solovyova and Verkhratsky 2003). The first attempts to induce Ca^{2+} release in single central neurons by caffeine or by metabotropic agonists induced only very slight $[Ca^{2+}]_i$ increases in a small number of neurons. Only after the cell was depolarized was a large $[Ca^{2+}]_i$ response observed (Murphy and Miller 1988; Irving *et al*. 1992; Shmigol *et al*. 1994; Irving and Collingridge 1998; Rae *et al*. 2000; Masgrau *et al*. 2001). This peculiar behavior may arise in dissociated neurons only, as application of caffeine to resting neurons in acute hippocampal slices, or stimulation of Ca^{2+} release from IP3-sensitive Ca^{2+} stores from pyramidal cortical neurons *in situ*, routinely evoked $[Ca^{2+}]_i$ responses without the requirement for a depolarizing pre-pulse (Garaschuk *et al*. 1997; Stutzmann *et al*. 2004). Nevertheless, dissociated neuronal cultures have proven invaluable for gaining an understanding of intracellular Ca^{2+} kinetics in neurons (Murphy and Miller 1988; Irving *et al*. 1992; Shmigol *et al*. 1994; Irving and Collingridge 1998; Rae *et al*. 2000; Masgrau *et al*. 2001). In essence, the 'empty' Ca^{2+} store within the ER favors Ca^{2+} accumulation upon depolarization, thus increasing the Ca^{2+} content within the store and permitting Ca^{2+} release (Verkhratsky 2005).

As for $PS1_{KI}$ and 3xTg-AD neurons, bath application of 25 mM caffeine to NonTg cortical neurons perfused with an extracellular solution containing 2.5 mM Ca^{2+} failed to evoke a rise in $[Ca^{2+}]_i$ (Fig. 2). Only after a conditioning depolarization was applied could stores accumulate Ca^{2+} and become available for discharge by caffeine (Shmigol *et al*. 1994). In agreement with this observation, bath application of 50 mM KCl evoked a rapid rise in $[Ca^{2+}]_i$ as Ca^{2+} enters via voltage-gated Ca^{2+} influx. Following removal of this depolarizing stimulus, $[Ca^{2+}]_i$ levels rapidly return to basal levels as Ca^{2+} is extruded from the cell predominantly via the plasma membrane, Na^+/Ca^{2+} exchanger (NCX) and Ca^{2+}-ATPase. $[Ca^{2+}]_i$ is also buffered and sequestered within the cell; for example, the ER Ca^{2+}-ATPase plays a role in maintaining low cytoplasmic Ca^{2+} levels by pumping Ca^{2+} ions into the ER. Following buffering and extrusion, application of caffeine evoked a rise of $[Ca^{2+}]_i$.

Basal Ca^{2+} levels (prior to a conditioning depolarization of 50 mM KCl) were not significantly different between NonTg, $PS1_{KI}$ and 3xTg-AD neurons (Fig. 3d). When neurons were perfused with a solution containing 2.5 mM Ca^{2+}, bath application of 50 mM KCl evoked a rapid rise in $[Ca^{2+}]_i$ due to Ca^{2+} entry through voltage-gated Ca^{2+} channels. The response of NonTg, $PS1_{KI}$ and 3xTg-AD neurons to this conditioning depolarization was not significantly different in terms of the magnitude of their peak response and rate of decay. Basal Ca^{2+} levels (prior to caffeine application) were not significantly different between NonTg, $PS1_{KI}$ and 3xTg-AD neurons (Fig. 3f). In NonTg neurons, when perfused with 2.5 mM Ca^{2+}

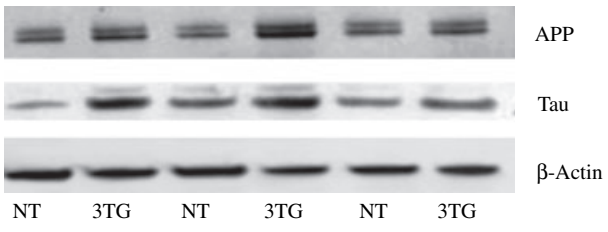

Fig. 1 Thy1.2-directed human APP and tau transgenes are expressed in dissociated embryonic neurons. APP and tau steady-state protein levels were monitored in protein extracts from E15 cortical cultures by western blots using 6E10 and HT7 antibodies. This experiment was performed in triplicate.

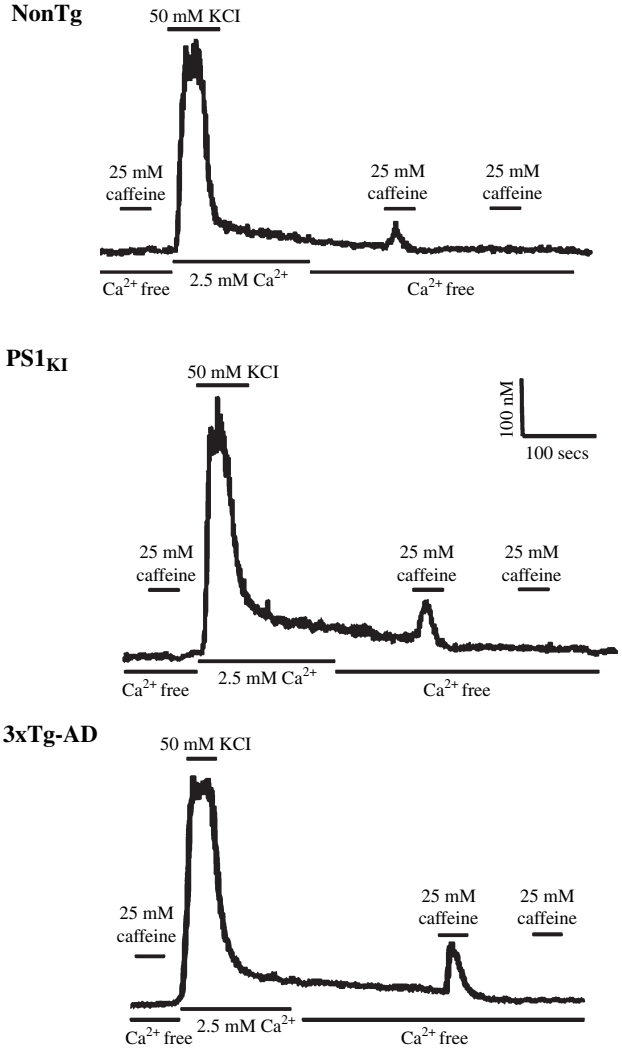

Fig. 2 Caffeine-sensitive Ca^{2+} stores are low in isolated NonTg, $PS1_{KI}$ and 3xTg-AD cortical neurons. Bath application of 25 mM caffeine fails to evoke a rise in Ca^{2+}. Following a conditioning depolarizing pulse of 50 mM KCl, 25 mM caffeine produces a rise in Ca^{2+}.

following a conditioning depolarization of 50 mM KCl, bath application of 25 mM caffeine caused a rapid transient rise in $[Ca^{2+}]_i$ that returned to basal levels with a time constant of 13 s (Fig. 3h). In contrast, in $PS1_{KI}$ and 3xTg-AD neurons, the peak rises of Ca^{2+} transients were significantly greater than those observed in NonTg neurons (Fig. 3g), although all these groups had similar time constants of decay (Fig. 3h). This finding suggests that Ca^{2+} stores are greater in both $PS1_{KI}$ and 3xTg-AD neurons, as calculated by the integral of the caffeine-induced Ca^{2+} transient signal (Fig. 3i). Notably, caffeine-evoked rises in $[Ca^{2+}]_i$ in both $PS1_{KI}$ and 3xTg-AD neurons were identical. Therefore, both transgenic mouse models display enhanced caffeine-induced Ca^{2+} responses that appear to be due to the M146V mutation in PS1 alone.

Ryanodine sensitivity of caffeine-induced Ca^{2+} release

To determine pharmacologically whether the observed responses were indeed mediated via the RyR, we next examined the effects of the RyR antagonist, ryanodine (2 μM). At submicromolar concentrations, ryanodine acts by binding to the RyR itself and stabilizes the channel in an open state or, at higher concentrations, by inhibiting opening of the RyR channel (Laporte *et al*. 2004).

Although ryanodine had no effect on voltage-gated Ca^{2+} entry, it did inhibit caffeine-induced rises in Ca^{2+} in all groups of neurons tested (Fig. 4). This inhibition required at least one caffeine exposure, consistent with the use-dependence of this drug (Fill and Copello 2002). We have also shown that another RyR antagonist, dantrolene (10 μM), can inhibit caffeine-induced Ca^{2+} release in NonTg, $PS1_{KI}$ and 3xTg-AD neurons as well (data not shown).

Ca^{2+} binding protein expression in $PS1_{KI}$ and 3xTg-AD cortex

There are a number of candidate proteins involved in $[Ca^{2+}]_i$ homeostasis that could, in part, account for the alterations in $[Ca^{2+}]_i$ seen in both the $PS1_{KI}$ and 3xTg-AD neurons reported here. Calbindin, calreticulin and calsenilin are all proteins whose expression levels are altered in AD (Taguchi *et al*. 2000; Palop *et al*. 2003; Jo *et al*. 2004). To address whether or not alterations in expression levels of these candidate proteins could potentially be responsible for the alterations in $[Ca^{2+}]_i$ observed in the $PS1_{KI}$ and 3xTg-AD neurons, western blots were used to compare their steady-state levels. It was clear that steady-state levels of these proteins were not significantly different between NonTg, $PS1_{KI}$ and 3xTg-AD mice (Fig. 5). Steady-state levels of the neuronal ER Ca^{2+} ATPase, SERCA-2B, were also unaltered. Therefore, the alterations in Ca^{2+} homeostasis are not likely to be attributable to alterations in the expression of these Ca^{2+}-binding proteins.

Ryanodine receptor expression in $PS1_{KI}$ and 3xTg-AD cortex

It has previously been reported that mouse hippocampal neurons expressing PS1 mutations exhibit greatly increased levels of RyR and enhanced Ca^{2+} release following stimulation with caffeine (Chan *et al*. 2000). Here, we report that neurons isolated from $PS1_{KI}$ and 3xTg-AD display enhanced caffeine-induced Ca^{2+} release following stimulation with caffeine, and that these responses are indistinguishable from each other (Fig. 3). This increase in cytosolic Ca^{2+} signal seen in both $PS1_{KI}$ and 3xTg-AD is consistent with the idea that RyR expression would be similarly elevated in both transgenic models. Again, we employed western blot using an antibody that recognizes all subtypes of the RyR to compare steady-state levels of RyR protein in NonTg, $PS1_{KI}$ and 3xTg-AD mice. We found that steady-state levels of RyR protein in $PS1_{KI}$ and 3xTg-AD cortex were significantly

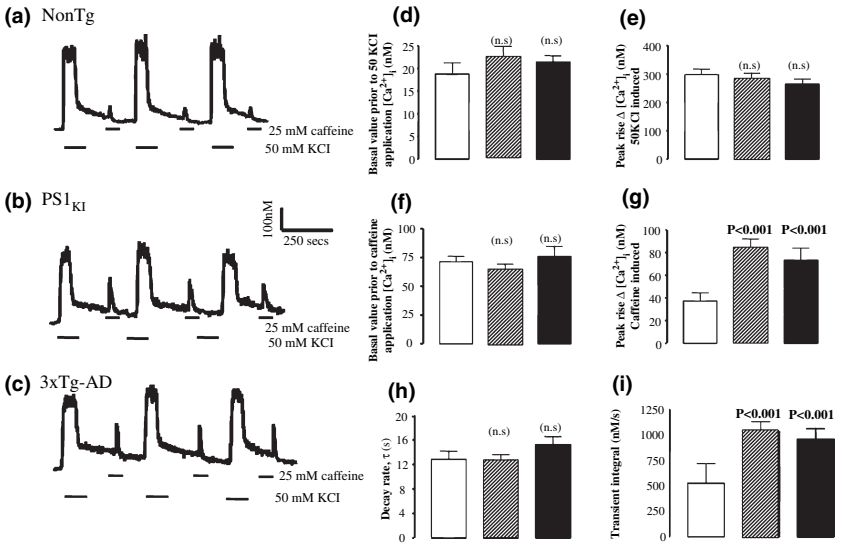

Fig. 3 Caffeine-sensitive Ca^{2+} stores are enhanced in neurons isolated from $PS1_{KI}$ and 3xTg-AD neurons. When depolarized briefly by a 50 mM KCl pre-pulse in a Ca^{2+}-containing medium, multiple responses to caffeine could be produced. Whilst voltage-gated Ca^{2+} entry was unaltered in 3xTg-AD, caffeine-induced rises of $[Ca^{2+}]_i$ were significantly increased in 3xTg-AD and $PS1_{KI}$ compared with NonTg neurons. Representative rises in $[Ca^{2+}]_i$ evoked by repetitive stimulation by bath application of 50 mM KCl followed by 25 mM caffeine in NonTg (a), $PS1_{KI}$ (b) and 3xTg-AD neurons (c). (d–i) Bar graphs (open bar: NonTG; hatched: $PS1_{KI}$; solid bar: 3xTg-AD) indicating mean values from parameters measured from recordings in (a), (b) and (c): basal $[Ca^{2+}]_i$ prior to 50 mM KCl (d), peak rise to 50 mM KCl (e), basal $[Ca^{2+}]_i$ prior to caffeine application (f), peak rise to 25 mM caffeine (g), decay rate (h) and transient integral of 25 mM caffeine induced rise in Ca^{2+} (i). $n = 21$ NonTG, $n = 18$ for $PS1_{KI}$ and $n = 25$ for 3xTg-AD.

increased compared with NonTg cells, and that the level of RyR protein in $PS1_{KI}$ and 3xTg-AD cortex was of a similar magnitude (Fig. 6). Caffeine induces Ca^{2+} release from RyR-sensitive Ca^{2+} stores, and both $PS1_{KI}$ and 3xTg-AD neurons show similar enhanced elevations in cytosolic Ca^{2+} following exposure to caffeine. One possible mechanism for this enhancement would be an enhanced release of Ca^{2+} via the increased number of RyR present in the ER, as reported here.

Discussion

The pathogenesis of AD is complex and involves many molecular and cellular pathologies. Disruption of $[Ca^{2+}]_i$ homeostasis is generally accepted to be a major factor contributing to neurodegeneration (LaFerla 2002). Ca^{2+} modulates a panoply of biological functions, so it is not surprising to find repeated suggestions that dysregulation of $[Ca^{2+}]_i$ homeostasis underlies many pathologies. In addition, many studies using primary cells from transgenic embryos have identified disturbances in Ca^{2+} signaling months before any obvious extracellular Aβ pathology. Consequently, alterations in $[Ca^{2+}]_i$ homeostasis appear to be an early event in the pathogenesis of AD.

Each cell type expresses a unique set of components that comprise what is now being called a Ca^{2+} signaling 'toolkit' consisting of, amongst other things, plasma membrane Ca^{2+}-ATPase, NCX, $[Ca^{2+}]_i$ buffering proteins, SERCA, mitochondria, etc. (Berridge *et al*. 2003). This toolkit results in Ca^{2+} transients with different spatial and temporal profiles depending on the influence each member of this toolkit plays. Ca^{2+} released from $[Ca^{2+}]_i$ stores is removed from the cell by various exchangers and buffers. The plasma membrane Ca^{2+} ATPase (PMCA) and NCX extrude Ca^{2+} to the outside, whereas the SERCA pumps Ca^{2+} back into the ER. Mitochondria also play a role in the recovery of a Ca^{2+} transient in that they sequester Ca^{2+} through a uniporter during a Ca^{2+} transient and release it more slowly back into the cytosol.

Our results suggest that Ca^{2+} stores in both the $PS1_{KI}$ and 3xTg-AD cortical neurons are elevated compared with NonTg cells. However, these results are complicated by the fact that the kinetics of transients rely not only on store size but also on Ca^{2+} extrusion mechanisms, buffering and release. Therefore, any situation in which the normal physiological functions of these extrusion mechanisms, buffering or release pathways are altered will have a profound impact on $[Ca^{2+}]_i$. For example, excessive production of reactive oxygen species, a likely candidate in the toxicity seen in AD, can damage membrane proteins and lipids, leading to enhanced $[Ca^{2+}]_i$ due to the dysregulation of membrane transporters important in the regulated movement of Ca^{2+} across the membrane. In addition, there is a growing body of evidence implicating abnormal mitochondrial function in AD (Varadarajan *et al*. 2000; Casley *et al*.

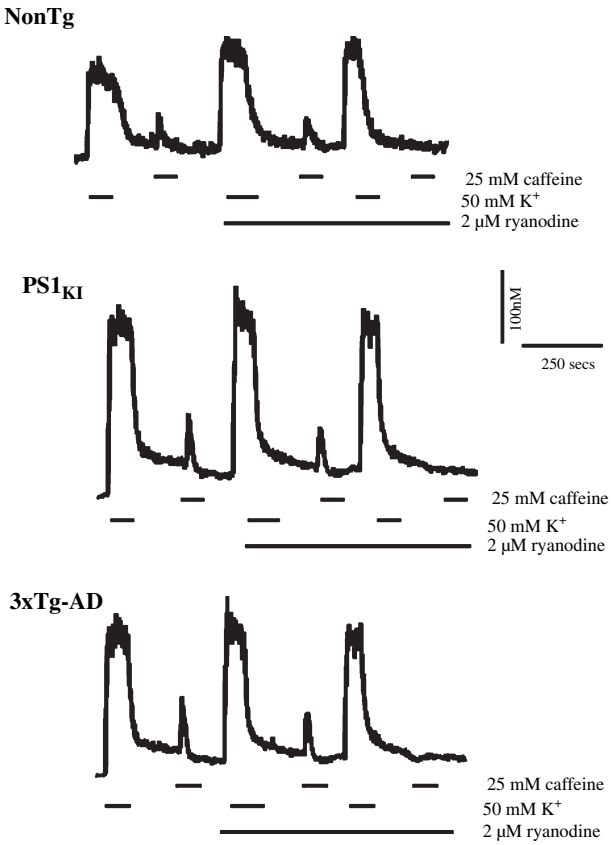

Fig. 4 Caffeine-sensitive Ca^{2+} stores in $PS1_{KI}$ and 3xTg-AD neurons are inhibited by ryanaline. Representative rises in $[Ca^{2+}]_i$ evoked by repetitive stimulation by bath application of 50 mM KCl, followed by 25 mM caffeine in NonTg, $PS1_{KI}$ and 3xTg-AD neurons. Ryanodine, whilst having no effect on voltage-gated Ca^{2+} entry, inhibited caffeine-induced rises in Ca^{2+} in NonTg, $PS1_{KI}$ and 3xTg-AD, requiring at least one caffeine exposure before inhibition was observed. $n = 18$ for NonTg, $n = 20$ for $PS1_{KI}$ and $n = 19$ for 3xTg-AD.

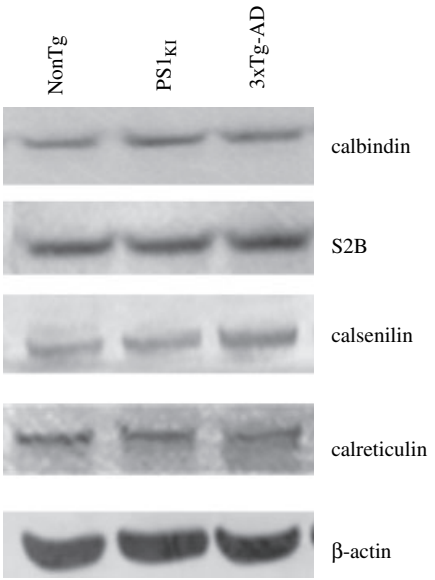

Fig. 5 Steady-state levels of candidate Ca^{2+}-binding proteins implicated in the pathogenesis of AD are not altered in $PS1_{KI}$ or 3xTg-AD mice. Example western blots illustrating that steady-state levels of calsenilin, calbindin D, calreticulin and SERCA-2B are comparable among the $PS1_{KI}$, 3xTg-AD and NonTg groups.

2002). It is not inconceivable then that in a pathological state such as AD, defects in any part of the Ca^{2+} signaling toolkit will have an untoward impact on a Ca^{2+} transient evoked by agonist stimulation. Therefore, relying on the integral of Ca^{2+} transient to 'predict' ER Ca^{2+} store size will disguise any influence that any member of this toolkit may have and provide an incorrect determination of supposed store size. It is unlikely, however, that Ca^{2+} extrusion mechanisms are affected in either the $PS1_{KI}$ or 3xTg-AD neurons as the rates of decay of the Ca^{2+} transients are similar between the NonTg, $PS1_{KI}$ and 3xTg-AD groups. Any defects in Ca^{2+} extrusion resulting from, for example, mitochondrial dysfunction would be expected to cause an alteration of this rate of decay.

Another mechanism that may account for the increase in $[Ca^{2+}]_i$ stores is altered expression of $[Ca^{2+}]_i$ buffering proteins. Calreticulin is a major Ca^{2+}-binding/buffering protein residing within the ER lumen. Calreticulin deficiency has been shown to lower ER luminal Ca^{2+} levels and protect cells from apoptosis, whereas overexpression of the protein in human embryonic kidney cells and mouse embryonic fibroblasts enhances ER Ca^{2+} levels and sensitizes cells to apoptosis (Groenendyk *et al*. 2004). One explanation for the enhancement in caffeine-induced Ca^{2+} release observed in both $PS1_{KI}$ and 3xTg-AD could simply be an enhancement in calreticulin levels, thereby providing storage for any increase in ER Ca^{2+} levels. However, this was not the case (Fig. 5). Neither were there alterations in the steady-state levels of two other Ca^{2+}-binding proteins implicated in the pathogenesis of AD, calsenilin and calbindin-D.

It has previously been shown that steady-state levels of RyR in $PS1_{KI}$ mice and PC12 cells expressing the $PS1_{KI}$ mutation are elevated (Chan *et al*. 2000). One consequence of an increase in the expression of this Ca^{2+} release channel would be an enhancement in the release of stored Ca^{2+}. It is clear that upon application of caffeine, evoked peak rises in $[Ca^{2+}]_i$ are significantly greater in both $PS1_{KI}$ and 3xTg-AD neurons compared with NonTg, and that these responses are identical to each other (Figs 3 and 4). Importantly, the decay rates of the Ca^{2+} transients are the same for NonTg, $PS1_{KI}$ and 3xTg-AD, suggesting that the clearance of $[Ca^{2+}]_i$ is not affected in the $PS1_{KI}$ and 3xTg-AD neurons. At present we cannot definitively provide reasons for the enhancement in caffeine responses observed here. It would seem unlikely that the enhanced response was due (solely) to increased ER Ca^{2+} levels as SERCA-2B and calreticulin levels were unaltered. However, we cannot rule out the possibility of altered

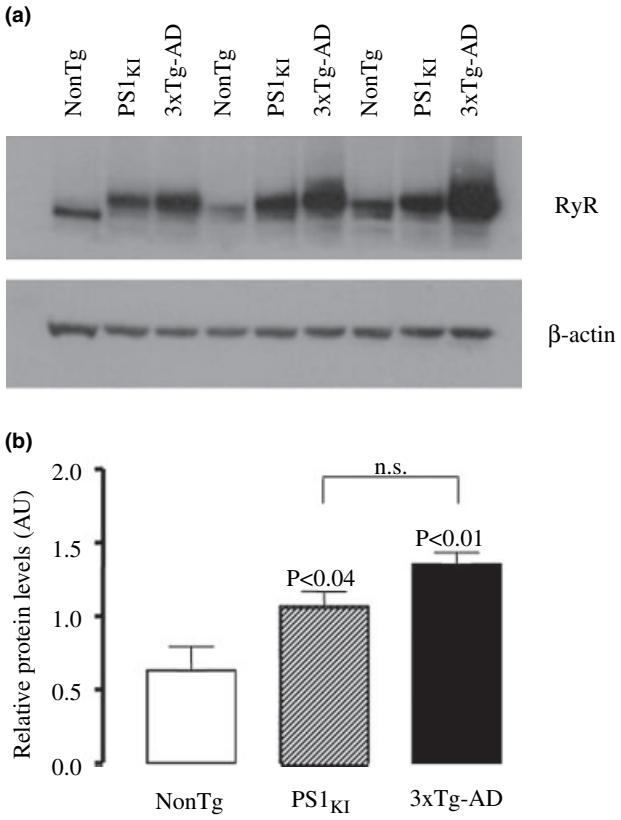

Fig. 6 Steady-state levels of RyR are increased in $PS1_{KI}$ and 3xTg-AD cortex. (a) Example western blots illustrating enhanced steady-state levels of RyR in $PS1_{KI}$ and 3xTg-AD compared with NonTg. (b) Averaged densiometric values relative to actin from experiments exemplified in (a); *p*-values above bars indicate statistically different values.

SERCA-2B activity. The increased expression of RyR reported in both $PS1_{KI}$ and 3xTg-AD neurons resulted in a faster release of stored Ca^{2+}. As the decay rates in NonTg, $PS1_{KI}$ and 3xTg-AD neurons were the same, it would take longer to clear the enhanced release of Ca^{2+} in both $PS1_{KI}$ and 3xTg-AD. To confirm this, future studies will focus on imaging intraluminal ER Ca^{2+} stores directly to assess properly the state of Ca^{2+} stores in both the $PS1_{KI}$ and 3xTg-AD neurons.

In conclusion, we have demonstrated enhanced caffeine-sensitive Ca^{2+} stores in the 3xTg-AD mouse model of AD. This enhancement of cytosolic Ca^{2+} signal was not attributable to defects in extrusion of Ca^{2+}, or to altered expression of a number of Ca^{2+}-binding proteins, but was associated with increased expression of the RyR. Interestingly, RyRs are abundantly expressed in brain regions prone to plaque accumulation in AD (hippocampal dentate gyrus, CA3/4 region and neocortex). Querfurth *et al.* (1997) have reported that the mobilization of a caffeine-sensitive RyR-regulated pool of Ca^{2+} accelerates Aβ production. Furthermore, a study by Kelliher *et al*. (1999) documented increased radiolabelled RyR binding in brain sections from the CA1 region of AD patients, raising the possibility that RyR expression levels, as well as pattern of expression, may be involved in the pathogenesis of this disease state.

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