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Impaired TNF- α Control of IP₃R-mediated Ca²⁺ Release in Alzheimer's Disease Mouse Neurons

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Abstract

The misguided control of inflammatory signaling has been previously implicated in the pathogenesis of several neurological disorders, including Alzheimer's disease (AD). Induction of tumor necrosis factor- α (TNF- α), a central mediator of neuroinflammation, occurs commensurate with the onset of early disease in 3xTg-AD mice, which develop both amyloid plaque and neurofibrillary tangle pathologies in an age- and region-dependent pattern. Herein, we describe regulation inherent to 3xTg-AD neurons, which results in the loss of TNF- α mediated enhancement of inositol 1,4,5 trisphosphate (IP₃R)-mediated Ca²⁺ release. This modulation also leads to significant down regulation of IP₃R signaling following protracted cytokine exposure. Through the experimental isolation of each AD-related transgene, it was determined that expression of the PS1^{M146V} transgene product is responsible for the loss of the TNF- α effect on IP₃R-mediated Ca²⁺ release. Furthermore, it was determined that the suppression of TNF- α receptor expression occurred in the presence of the presenilin transgene. Our findings attribute this familial AD mutation to suppressing a Ca²⁺-regulated signal cascade potentially intended to “inform” neurons of proximal neuroinflammatory events and trigger compensatory responses for protection of neural transmission.

Keywords

Alzheimer's disease; β -amyloid; tau; presenilin; 3xTg-AD; neuroinflammation; calcium homeostasis

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1. Introduction

Altered Ca^{2+} signals have been shown previously to occur in various animal models of Alzheimer's disease (AD). Specifically, two of the transgenes expressed by the 3xTg-AD mouse, a murine model of AD that develops amyloid and tau-related pathologies in age- and region-dependent patterns, have been linked to dysfunctions in Ca^{2+} handling. First, the human amyloid precursor protein-Swedish (hAPP^{swe}) mutation was found to affect the basal cytosolic [Ca^{2+}] in primary cortical neurons from hAPP^{swe} and 3xTg-AD mice [1]. Through pharmacological methods, this enhancement was attributed to both altered voltage-gated Ca^{2+} entry and increased inositol 1,4,5 trisphosphate (IP_3R)-mediated Ca^{2+} release. Additionally, it has been demonstrated that the hAPP^{swe} mutation in the TgCRND8 mouse model underlies increases in Ca^{2+} -activated K^+ channel activity in hippocampal slices, again suggesting dysfunctional Ca^{2+} handling arises when the hAPP^{swe} transgene is neuronally expressed [2].

Secondly, the presenilin-1 M146V (PS1^{M146V}) familial AD mutation also regulates several cellular processes that promote altered Ca^{2+} release. Wild-type presenilin (PS^{WT}) serves as a key subunit of the γ -secretase complex that participates in proteolytic processing of APP. In addition, PS^{WT} has also been proposed to act as a component of an endoplasmic reticulum (ER) channel that protects against high levels of ER Ca^{2+} by facilitating a passive, constitutive Ca^{2+} leak. In planar lipid bilayers and mouse embryonic fibroblast cultures, the PS1^{M146V} mutation eliminates ion permeability, suggesting that this autosomal-dominant mutation abolishes the normal function of the channel [3,4]. PS1^{M146V} has also been suggested to alter RyR subtype 2 expression, thereby enhancing Ca^{2+} -induced Ca^{2+} release [5,6]. Moreover, this mutation has been shown to modulate IP_3R -mediated Ca^{2+} release in cortical neurons and *Xenopus oocytes* by directly interacting with the IP_3R to alter its open probability [7-10]. Lastly, as a subunit within the γ -secretase complex, PS1^{M146V} expression results in enhanced proteolytic liberation of the pathogenic peptide amyloid-beta 1-42 ($\text{A}\beta$ 1-42). Oligomerization of this hydrophobic peptide and potential insertion into the plasma membrane may facilitate passive entry of Ca^{2+} in neurons, ultimately leading to dysregulated Ca^{2+} homeostasis [11].

Coincident with these physiologic processes in the setting of AD is the marked enhancement of proinflammatory signaling related to cytokine, chemokine, and complement protein release by microglia, astrocytes, oligodendroglia, and neurons. Prolonged neuronal exposure to the potent cytokine, tumor necrosis factor- α (TNF- α) has been shown to enhance voltage-gated Ca^{2+} channel conductance [12]. Additionally, TNF- α exposure alters the activity of two different glutamatergic receptors. First, a subset of Ca^{2+} permeable AMPA receptors lacking the GluR2 subunit have been previously shown to be rapidly inserted into the plasma membrane after cytokine addition [13,14]. Second, the application of TNF- α leads to the down regulation of NMDA currents, another glutamate-activated Ca^{2+} permeable channel [12]. Electrophysiological studies have corroborated these findings, illustrating that TNF- α exposure decreases long-term potentiation (LTP), a electrophysiological correlate to learning and memory behavior [15]. Furthermore, mice deficient in cognate TNF- α receptor expression have also been shown to display no long-term depression (LTD) in response to Schaffer collateral pathway stimulation, due to a lack of NF κ B activation [16].

Although inflammatory processes are believed to arise in parallel with alterations in intraneuronal Ca^{2+} handling in AD, the role of key proinflammatory mediators in the generation and/or modulation of these Ca^{2+} signaling defects in the context of AD-related gene expression has yet to be fully elucidated. We previously demonstrated that 3xTg-AD mice develop a brain region- and age-associated increase of TNF- α and microglial activation [17]. Moreover, we have observed enhanced agonist-induced Ca^{2+} signals in these mice, consistent with previously published reports [5,6]. To that end, we postulated enhanced TNF- α signaling

could exacerbate the already augmented IP₃R-derived Ca²⁺ signals in 3xTg-AD neurons [18], potentially contributing to neuronal demise observed in human AD. Surprisingly, we found that TNF- α signaling was markedly down regulated in 3xTg-AD neurons, resulting in the inability of exogenously applied cytokine to regulate IP₃R signaling. This suppressive mechanism, which was determined to be PS1^{M146V}-dependent, may make AD-afflicted neurons more susceptible to disease-related insults. Furthermore, TNF-R down regulation may diminish the ability of these neurons to properly respond to the surrounding neuroinflammatory environment and to initiate critical Ca²⁺-driven protective responses *in vivo*.

2. Materials and methods

2.1 Cell culture

Primary neuronal cultures and Neuro2A cells (ATCC, Manassas, MA) were cultured as previously described [19]. Briefly, primary neurons were obtained by microdissection of 3xTg-AD E_{14.5} cortices, trypsin digestion, and subsequent plating on polyethyleneimine (PEI; Sigma, St Louis, MO) coated coverslips. B-27 supplemented Neurobasal Media (Invitrogen, Carlsbad, CA) was changed every four days. Neuro2A cells were cultured between passages 2-20 in DMEM (Invitrogen) with 8% fetal bovine serum (Gemini, West Sacramento, CA). Neuro2A cells were separately transfected with eukaryotic expression plasmids (pCDNA3) harboring cDNAs expressing hAPP^{swe}, tau^{P301L}, PS1^{WT}, or PS1^{M146V} (all kindly provided by D. W. Van Nostrand, SUNY-Stony Brook). Two days later, cells were placed under 400 μ g/ml G418 (Invitrogen, Carlsbad, CA) for 2-3 weeks. Single stable cell clones for each construct were isolated using cloning cylinders and subsequently screened by qRT-PCR for expression of appropriate AD-related transgene.

2.2 Ca²⁺ imaging

Neuro2A cells/clonal lines or primary neurons were plated on glass coverslips (Carolina, Burlington, NC) in 12-well culture dishes (Corning, Corning, NY). Before imaging, 100ng/mL of murine recombinant TNF- α or PBS, as a negative control, was added to the culture media. Cells were loaded with 1 μ M of the Ca²⁺-sensitive dye Fura2-AM (TEFLABS, Austin, TX) for 15 min. at 37°C in a physiologically-based salt solution (PBSS) containing in mM: 5.5 glucose, 0.56 MgCl₂, 2 KCl, 1 Na₂HP0₄, 10 HEPES, and 1.2 CaCl₂, pH 7.4. Coverslips were mounted in an imaging chamber and transferred to an inverted Nikon microscope with a 40x oil immersion objective lens (numerical aperture, 1.3). Addition of bradykinin (100nM, Sigma), caffeine (25mM, Sigma), or CCh (25 μ M, Sigma) diluted in PBSS to elicit a Ca²⁺ response was accomplished using a gravity-fed system (Warner Instruments, Camden, CT) while 340 / 380 nm excitation was provided by a Polychrome V monochromator illumination system under the control of TILL Vision software. A SENSICAM-QE (Cooke, Leicester, UK) was used to detect 510-nm emission from 20-ms exposures at a sampling rate of 1 Hz. Reciprocal increases in fluorescence from 340-nm excitation and decreases in 380-nm excitation fluorescence corresponded to an increase in intracellular Ca²⁺. During agonist addition, the average deviation from baseline was calculated for each neuron and these data were then pooled for each coverslip (n>8 for each condition). A change of 2 standard deviations from baseline was considered a viable response. Mathematical and statistical analyses of the Ca²⁺ signals were performed using Graph Pad Prism software.

2.3 Quantitative real-time RT-PCR and Western blotting

RNA was purified from 3xTg-AD neuronal cultures using the TRIzol (Invitrogen) phenol-chloroform method. A high-capacity cDNA archiving kit (Applied Biosystems, Foster City, CA) was utilized to convert 2 μ g of RNA into cDNA. Transcript levels were quantified utilizing an Assay-on-Demand primer probe set (Applied Biosystems) specific to the type-1 mIP₃R,

hPS1, hAPP, hTau, or mTNF-RI / RII transcripts. Normalization was accomplished utilizing a 18s rRNA-specific primer/probe set. Protein levels of each TNF receptor were quantified by Western blotting, where Neuro2A cell monolayers were lysed in a modified RIPA buffer (50mM Tris-HCL, 1% NP-40, 150mM NaCl, 0.25% Nadeoxycholate, 1mM EDTA, pH 7.4) containing 1X Protease Inhibitor Cocktail (Sigma) and homogenized through a 21.5-gauge needle. The lysates were then subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was then incubated with TNF-RI or TNF-II-specific antibodies (1:1000, AbCam), and β -actin (1:2000, Sigma) antibodies for 2h. Affinity-purified, horseradish peroxidase (HRP)-conjugated Donkey anti-rabbit/mouse antibodies were subsequently applied (1:5000, Sigma) in combination with Western Lightning reagent (Perkin Elmer, Waltham, MA) to visualize immunoreactive receptor species. Band intensities were quantified using Labworks software and presented as OD ratios of TNF-RI or TNF-II to β -actin.

2.4 IP₃R promoter-driven reporter plasmids

The type-1 human IP₃R promoter construct was kindly provided by Dr. Peter Sims (University of Rochester) [20]. This constructs contained a defined segment of the IP₃R promoter inserted into the pGL3 vector (Promega, Madison, WI) upstream of the firefly luciferase reporter gene. Transient transfection of this construct with a nucleofection system (Amaxa, Gaithersburg, MD) and subsequent activation was provided by TNF- α and a constitutively active cJun N-terminal kinase-kinase, Δ MEKK1 (Dirk Bohmann, University of Rochester). Luciferase activity was measured from the Neuro2A cell lysates using the Dual-Luciferase® Reporter assay (Promega) in accordance with the manufacturer's directions. A Lumicount luminescent plate reader (Packard, Downers Grove, IL) was first used to monitor firefly luciferase activity and then *Renilla* luciferase activity after the addition of a Stop and Glo® reagent (Promega). *Renilla* expression was used as an internal transfection control, levels of which remained unchanged in each of the transfection experiments.

2.5 siRNA-mediated knockdown

Mouse TNF-RI and TNF-II-specific siRNA-expressing plasmids and scrambled control constructs were designed previously by Wade Narrow (University of Rochester), synthesized by InvivoGen (San Diego) and validated by Ms. Sara Montgomery (University of Rochester). Neuro2A cells were nucleofected with psiRNA-7SKneo-mTNFRSF1a for TNF-RI knock-down, psiRNA-7SKneo-mTNFRSF1b for TNF-II knock-down, or psiRNA-7SKneo-mTNFscr as a control and 72h later, cells were fixed with 4% PFA. Immunocytochemistry was subsequently performed using receptor-specific antibodies (AbCam) and FITC-conjugated secondary antibodies (Sigma). Images were captured using a confocal microscope (Olympus, Center Valley, PA). Subsets of siRNA-nucleofected cultures were treated with TNF- α and Ca²⁺ imaging studies conducted as described above.

3. Results

3.1 TNF- α fails to enhance carbachol-mediated Ca²⁺ signals in 3xTg-AD neurons

Primary neuronal cultures from 3xTg-AD mice were obtained through the dissection and plating of the neuronal cortices of E_{14.5} pups. Ca²⁺ imaging was performed with the ratiometric Ca²⁺ indicator, Fura2-AM, while the stable acetylcholine agonist carbachol (CCh) provided muscarinic stimulation. An exposure of 25 μ M CCh, a sub-maximal concentration of agonist, to the 3xTg-AD neurons elicited a response in approximately 50% of 3xTg-AD neurons in the field of view, indicating that any potential regulation of muscarinic signaling following TNF- α exposure could be readily monitored (Fig. 1A). Twenty-four hours after the addition of TNF- α , no significant alteration in the Ca²⁺ signals after CCh stimulation was observed in the 3xTg-AD neuronal cultures (Fig. 1B). Furthermore, in stark contrast to that which was observed previously with non-transgenic C57BL/6 neurons [18], 48h of TNF- α treatment led to a

significant decrease in the response of the 3xTg-AD neurons to CCh (Fig. 1B). This reduced trend of intracellular Ca^{2+} signals elicited by CCh addition was maintained at the 72-h time point, indicating that C57BL/6 and 3xTg-AD primary neurons respond differently to CCh when exposed to $\text{TNF-}\alpha$.

We previously reported that $\text{TNF-}\alpha$ exposure does not alter Ca^{2+} clearance in C57BL/6 neurons [18]. However, since presenilins can regulate SERCA pump function [21], we examined the ability of $\text{TNF-}\alpha$ or the expression of the 3xTg-AD transgenes to alter Ca^{2+} clearance after caffeine stimulation. Although $\text{TNF-}\alpha$ exposure did not affect Ca^{2+} clearance in 3xTg-AD neurons (Fig. 1C), significantly lower decay time constants were detected in 3xTg-AD neurons (14.8 ± 1.3 tau seconds) as compared to those exhibited by C57BL/6 neurons (26.3 ± 6.5 tau seconds), suggesting higher Ca^{2+} clearance activity in the former.

3.2 Quantitative real-time RT-PCR reveals decreased type-1 IP_3R mRNA levels in 3xTg-AD neurons following $\text{TNF-}\alpha$ exposure

Even though the IP_3R -mediated Ca^{2+} signals in 3xTg-AD neurons were unaffected by $\text{TNF-}\alpha$ exposure, we sought to determine if the reduction in CCh-induced Ca^{2+} signals at the 48-h time point correlated with a reduction in steady-state type-1 IP_3R mRNA levels. If a reduction in mRNA levels did occur, it would further establish the link between type-1 mRNA levels and IP_3R -mediated Ca^{2+} release previously observed in non-transgenic mouse neurons [18]. Messenger RNA was purified from untreated and $\text{TNF-}\alpha$ treated 3xTg-AD primary cortical neurons and real-time quantitative RT-PCR was performed using a primer/probe set specific for mouse type-1 IP_3R . A significant decrease in type-1 IP_3R transcripts was observed in $\text{TNF-}\alpha$ treated 3xTg-AD neurons at the 24-h time point, and recovered to control levels by the 48- and 72-h time points (Fig. 2). The loss of the enhancement signal and the addition of a suppressive signal suggested that at least two alterations were occurring in the response of the 3xTg-AD neurons to $\text{TNF-}\alpha$. Furthermore, since the 3xTg-AD neurons harbor three separate transgenes, it was unclear which of the AD-related transgene product(s) was/were ultimately responsible for these alterations.

3.3 PS^{M146V} is responsible for the altered response to $\text{TNF-}\alpha$

To implicate the transgene product(s) ultimately responsible for the alteration in $\text{TNF-}\alpha$ mediated modulation of IP_3R expression and downstream ER Ca^{2+} levels, we generated neuronal cell lines that individually expressed the 3xTg-AD transgenes: hAPP^{swe} , $\text{PS1}^{\text{M146V}}$, or $\text{tau}^{\text{P301L}}$. Neuro2A cells, which were previously shown to harbor a pathway to similar mouse primary neurons for $\text{TNF-}\alpha$ driven potentiation of IP_3R -mediated Ca^{2+} signaling, were selected for the analysis of individual transgene function [19]. G418-resistant clones were tested for transgene expression using quantitative real-time RT-PCR and clones expressing their respective transgene at similar levels were selected for further study (Fig. 3A).

The ability of $\text{TNF-}\alpha$ to modulate IP_3R -mediated Ca^{2+} release was assayed in each AD transgene-expressing Neuro2A cell line [19]. In Neuro2A cells expressing the hAPP^{swe} and $\text{tau}^{\text{P301L}}$ transgenes, the extent of Ca^{2+} release following bradykinin addition was significantly enhanced with a 24-h $\text{TNF-}\alpha$ exposure (Fig. 3B). This increase in IP_3 -derived Ca^{2+} signals was reminiscent of that observed in C57BL/6 neurons and wild-type Neuro2A cells, suggesting that these transgenes were not individually responsible for the alteration in the response to the cytokine [18]. In contrast, Neuro2A cells stably expressing $\text{PS1}^{\text{M146V}}$ did not produce augmented responses following $\text{TNF-}\alpha$ exposure, implicating presenilin in the unique 3xTg-AD neuron-specific response (Fig. 3B).

Furthermore, we examined if the modulation was inherent to the $\text{PS1}^{\text{M146V}}$ mutation, by comparing the response of stably transfected $\text{PS1}^{\text{M146V}}$ Neuro2A cells to that engendered in

stably transfected PS1^{WT} Neuro2A cells. Again, individual Neuro2A cell clones expressing similar levels of PS1 gene transcripts, as indicated via qRT-PCR, were utilized (Fig. 3A). Neuro2A cells stably transfected with the PS1^{WT} gene exhibited enhanced IP₃R-mediated Ca²⁺ release following cytokine exposure (Fig. 3C). In contrast, the overexpression of the PS1^{M146V} gene blocked the potentiation in IP₃R release, suggesting the mutation was indeed responsible for the alteration in the TNF- α signaling pathway in 3xTg-AD neurons (Fig. 3C).

In addition to the lack of response at the 24-h time point, the 3xTg-AD neurons exhibited a significant reduction in IP₃R-mediated Ca²⁺ release 48h after TNF- α addition (Fig. 1B). The PS1^{M146V} stably transfected Neuro2As were examined at 24, 48, and 72h post-cytokine addition to determine if this response was also due to mutated presenilin expression. Bradykinin-induced Ca²⁺ signals were significantly decreased at the 48-h time point, further indicating the importance of this mutation in the altered 3xTg-AD response (Fig. 3D).

3.4 PS1^{M146V}-induced alteration in Ca²⁺ signals lies upstream of JNK activation

The Ca²⁺ imaging data indicated an alteration in the regulation of IP₃R by TNF- α both in PS1^{M146V} Neuro2A cells and 3xTg-AD neurons. Previously, we discovered a c-Jun N-terminal kinase (JNK) dependent, SP-1 mediated regulation of the IP₃R promoter in Neuro2A cells [19]. Promoter activity was shown to be regulated by both a construct that directly activated JNK (Δ MEKK1) and by TNF-R activation with TNF- α [19]. Using this experimental paradigm, the level at which the alteration in the pathway occurred could be readily tested. Luciferase assays indicated that only Δ MEKK1 was able to significantly enhance full-length IP₃R promoter activity in the presence of PS1^{M146V} expression (Fig. 4). These data indicated that the alteration in the TNF- α pathway occurred at or above the level of JNK activation.

3.5 Both TNF-R subtypes can initiate the TNF- α /IP₃R pathway

We next assessed the respective contributions of the major TNF-R subtypes, TNF-R I and TNF-R II, in this pathway. Immunocytochemistry (ICC) illustrated the expression of both TNF-R I and TNF-R II in Neuro2A cells (Fig. 5 B,F). Moreover, nucleofection with plasmids expressing TNF-R subtype specific siRNAs reduced receptor expression at the protein level 48h after introduction (Fig. 5 D,H), whereas a control (scrambled) siRNA was without effect (Fig. 5 C,G).

By utilizing these siRNA constructs, we sought to determine the receptor(s) responsible for the modulation of IP₃R signaling. As predicted, cultures receiving the scrambled siRNA control construct exhibited enhanced bradykinin-induced Ca²⁺ signals after cytokine exposure (Fig. 5I). Treatment with a siRNA construct targeted against either receptor subtype, leaving the other receptor to signal following TNF- α engagement, again resulted in elevated Ca²⁺ signals in response to TNF- α exposure (Fig. 5I). However, the introduction of both TNF-R I and TNF-R II siRNA plasmids resulted in no change in bradykinin-induced Ca²⁺ signals (Fig. 5I), indicating that the cytokine could mediate its effects through either of the TNF- α receptor subtypes [18,19].

3.6 TNF-R expression is significantly reduced in cells harboring the PS1^{M146V} AD transgene

Since a down regulation of TNF-R expression could be responsible for the loss of cytokine effect in PS1^{M146V}-transfected Neuro2A cells and 3xTg-AD neurons, the expression of the TNF-Rs was assessed at both the mRNA and protein levels. Steady-state levels of both TNF-R I and TNF-R II mRNAs were down regulated in PS1^{M146V}-expressing Neuro2A cells (Fig. 6A). In addition, a significant down regulation of TNF-R I (p=0.022) and TNF-R II (p=0.028) protein in cell lysates generated from Neuro2A cells stably transfected with PS1^{M146V} was also observed (Fig. 6B,C). Furthermore, Western blot analysis of 3xTg-AD neuronal protein lysates indicated a similar down regulation in TNF-R I protein levels as compared to C57BL/

6 neurons (Fig. 6D), arguing that PS1^{M146V} mediates the TNF- α signaling defect in 3xTg-AD neurons by modulating TNF-R expression. The levels of TNF-RII protein in primary neurons of either genotype were below the level of detection, a finding that differentiates them from Neuro2A cells, and possibly indicates TNF-RII does not play a major role in TNF- α signaling within neuronal cultures.

4. Discussion

The pro-inflammatory cytokine, TNF- α has been shown previously to modulate IP₃R-mediated Ca²⁺ release through regulation of IP₃R expression in C57BL/6 neurons and wild-type Neuro2A cells [18,19]. This mechanism arises independently of alterations in Ca²⁺ entry, Ca²⁺ sequestration, and Ca²⁺-induced Ca²⁺ release. Given these previously reported findings, the long-term physiological consequences of dynamic TNF- α mediated control over IP₃R expression and Ca²⁺ homeostasis have yet to be determined. As we previously postulated, the enhancement of muscarinic signals in “normal” neurons could represent a single step in a process of preparing the cell for programmed death. The presence of a secondary inflammatory signal, for instance an excitatory amino acid, could result in neuronal death. If this is the case, the enhancement of IP₃R steady-state levels could facilitate the changes in Ca²⁺ signals necessary for initiation of apoptosis. The effect of such an enhancement in Ca²⁺ release has been previously shown to result in decreased cellular viability [22].

Conversely, we propose a model illustrated in Fig. 7 to explain one possible physiologically important effect of this pathway. Our model demonstrates how cytokine-induced increases in IP₃R expression could provide a physiological “brake” to the excitotoxicity present from excessive neurotransmitter release. CNS damage may result in disproportionate neuronal stimulation. Neurons containing the TNF- α /IP₃R pathway can up-regulate IP₃R-mediated Ca²⁺ release which would then lead to enhanced Ca²⁺-activated K⁺ channel activity. This hyperpolarizing force could stop the progression of an excitotoxic insult and save the downstream neuronal circuitry. In neurons expressing the AD-related PS1^{M146V} mutant transgene, the lack of this pathway would result in the continued spread of the apoptosis-inducing signal due to its suppression of TNF-R expression. This process would also leave these cells in an active state and inherently more susceptible to other excitotoxic insults associated with a neurodegenerative microenvironment, leading to the death of neurons close to the insult and a furthering of CNS dysfunction.

Our data indicate that differences in TNF-R subtype expression may indicate why certain neuronal populations are more at risk for cellular dysfunction and demise in neurological diseases exhibiting underlying chronic neuroinflammation. Experiments designed to selectively introduce exogenous TNF-R sub-types and binding partners into PS1^{M146V} Neuro2A cells and 3xTg-AD neurons could be used to establish specific roles for each participant in the signaling cascade. Through these studies a better understanding of the role TNF- α plays in neuroinflammatory pathology will be obtained.

The mechanism responsible for the down regulation of TNF-R expression in cells expressing the PS1^{M146V} transgene is currently unknown, but previously published literature provides potential insight. Others have shown that the presenilin gene, in association with other factors comprising the gamma-secretase complex, can cleave the p75 neurotrophic factor receptor (TNF-R^{NTF}, a TNF receptor family member) intramembraneously [23,24]. This cleavage results in less surface-bound TNF-R^{NTF} protein and possibly a down regulation in TNF-R^{NTF} expression, as the cleavage product has been further suggested to alter gene expression and neuronal viability [25]. A similar process may exist with TNF-RI and TNF-RII, where PS1^{M146V} alters the proteolytic processing of the receptors, thereby leading to diminished cell-associated TNF-R. In fact, preliminary evidence for this process has been illustrated by Harte

and colleagues [26]. This group reported that TNF-RI represents a novel substrate for presenilin-dependent regulated intramembrane proteolysis and postulates that presenilins play a central role in TNF-RI-mediated signaling. It is conceivable given the effect of the PS1^{M146V} mutation on γ -secretase-mediated processing of APP that such a mutation may alter the efficiency by which at least the TNF-RI subtype is processed intramembraneously. This could lead to enhanced shedding of the receptor, thereby inherently diminishing the TNF- α responsivity of the neuron. It is also possible that since gamma-secretase participates in the proteolysis of numerous substrates, a multitude of other signaling pathways may be involved. As an additional possibility, an enhancement in TNF- α expression by glia or by neurons themselves could play a role in TNF-R down regulation, since several groups have reported that an enhancement in TNF- α signaling can negatively impact TNF-R expression via a feedback loop [27,28].

The regulation of Ca²⁺ clearance associated with PS1^{M146V} expression was also observed in the present study. With the examination of presenilin-mediated modulation of SERCA function still in its infancy, only a basic understanding of the protein-protein interaction is currently known [21]. It will be of significant interest to the field of AD research to elucidate the mechanism by which mutations in PS1 affect SERCA pump activity or Ca²⁺ leak from the ER, since both processes have been hypothesized to alter the generation of fibrillogenic amyloid-beta peptides [3,21].

5. Conclusion

Elucidation of the cell types that differentially respond to neuroinflammatory insults via the TNF- α /IP₃R pathway *in vivo*, in both wild type and murine models of neurodegeneration, may hold the key for understanding the pathophysiological relevance of this novel pathway. Future studies examining this phenomenon *in vivo*, may indicate that this pathway plays a protective role and its suppression by mutant PS1-mediated down regulation of TNF-R expression may make a subset of neurons more vulnerable to an inflammatory insult. Moreover, neurons isolated from specific brain sub-regions, which are known to be especially vulnerable to AD pathology (i.e., hippocampal neurons), may serve as a more informative population in which to assess dysfunction in the TNF- α /IP₃R pathway. To this end, further experimental analysis of this signaling cascade is warranted. Studies designed to elucidate a better understanding of how familial AD mutations regulate ER Ca²⁺ levels in *in vivo* models of the disease have the potential to uncover the true pathophysiologic origin and role of Ca²⁺ signaling dysfunctions in the initiation and/or progression of late-onset sporadic AD.

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Figure 1. The effect of TNF α on IP₃R-mediated Ca²⁺ signals in 3xTg-AD neurons
 CCh (25 μ M) was utilized to examine IP₃R-mediated Ca²⁺ signals at 24, 48, and 72h after cytokine addition. (A) The percentage of responding cells in each field of view is shown for the 25 μ M dose of CCh. (B) Primary 3xTg-AD neuronal cultures were examined for alterations in IP₃R-mediated CCh signals at 24, 48, and 72h after TNF- α addition. Each diamond indicates the average peak response from baseline from on average 8 neurons per coverslip. Small boxes indicate the mean, large boxes indicate one standard error of the mean, and whiskers represent one standard deviation from the mean. (C) The decay of caffeine (25mM) induced Ca²⁺ signals were fit to a single exponential. Average decay constants (tau) for TNF- α and PBS treated neurons are indicated. p values were obtained from two-tailed student's T-testing.

Figure 2. Expression of the type-1 IP₃R following TNF- α treatment

Total RNA was extracted from DIV 12,13,14 (24-72h post TNF- α exposure) 3xTg-AD neuronal cultures by the TRIzol / phenol-chloroform gradient method and cDNA was generated using the High-Capacity cDNA Archive Kit. A primer-probe set specific for the type-1 IP₃R was used to detect mRNA levels with normalization to 18s rRNA. A significant decrease ($p < 0.05$) was observed in IP₃R mRNA 24h post TNF- α addition.

Figure 3. Stably expressing PS1^{M146V} Neuro2A cells display the same response to TNF- α as 3xTg-AD primary neurons

Neuro2A cells were stably transfected with each of the transgenes from the 3xTg-AD Alzheimer's disease mouse model. The expression of PS1^{M146V}, PS1^{WT}, APP^{SWE}, and tau^{P301L} in their respective stable cell lines was examined with specific primer / probe sets via qRT-PCR. (A) An increase in expression over wild-type Neuro2A (non-transfected) is shown for each of the clones. (B) Neuro2A cells were loaded with the Ca²⁺ indicator dye Fura2-AM and 100nM bradykinin was used to stimulate IP₃R-mediated Ca²⁺ release. Peak Ca²⁺ response after 24h treatment with 100ng/mL TNF- α for PBS is shown for each of the stably transfected cell lines. (C) The necessity of the M146V mutation was studied by comparing the response

to TNF- α in wild-type PS1 and PS1^{M146V} expressing Neuro2A cells lines. (D) The effect of the cytokine on IP₃R-mediated Ca²⁺ release in PS1^{M146V} Neuro2A cells is shown at the 24, 48, and 72-h time points. Box plot parameters: The center of the box is the mean of the coverslip averages, which are represented by the closed circles, box edges are the standard error of the mean, and the whiskers are the standard deviation of the mean. “*” represents p values <0.05 obtained by two-tailed Student's t-testing.

Figure 4. IP₃R promoter regulation in PS1^{M146V} stably transfected Neuro2As

A full-length IP₃R promoter luciferase reporter construct was transiently transfected into Neuro2A cells stably expressing PS1^{M146V} and the cells were treated with TNF- α or cotransfected with Δ MEKK1. Enhanced luciferase activity with Δ MEKK1 suggests its activation of the IP₃R promoter. Error bars represent one standard error of the mean.

Figure 5. siRNA-mediated knockdown analysis suggests either TNF-R subtype can initiate IP₃R potentiation

Immunocytochemistry (ICC) was performed to determine the expression pattern of the two major TNF- α receptor subtypes in cultured Neuro2A cells. (B-H) Confocal microscopy of the TNF-R subtypes showed expression of the receptors in Neuro2A cells, in the absence and the presence of siRNA constructs specifically targeted to each of the receptors. (I) siRNA constructs and a scrambled control construct were used in conjunction with Ca²⁺ imaging to determine which receptor was responsible for the TNF- α IP₃R expression pathway. *: $p < 0.05$ by one-way ANOVA and a Bonferroni post-hoc test.

Figure 6. Down regulation of TNF-R expression with PS1^{M146V} Expression

The expression of TNF-R I and II was examined in Neuro2A cells stably transfected with PS1^{M146V}. (A) mRNA from stably transfected Neuro2A cells was isolated by the phenol/chloroform extraction method and archived into cDNA. Primer probe sets specific for the TNF-R subtypes were utilized with normalization to the 18sRNA gene. (B) TNF-R expression in stably transfected wild-type and M146V presenilin 1 Neuro2A cells was monitored via Western blotting. (C) OD measurements are shown for quantifying the intensity of the bands in B. Additionally, expression of the type-1 TNF R was assayed in protein lysates derived from C57BL/6 and 3xTg-AD neurons. (D) TNF-R I expression was determined through SDS-PAGE

and Western blotting techniques. The blot was subsequently stripped and incubated with a β -actin specific antibody to assess gel-loading variability.

C57BL/6 **3xTg-AD**

Figure 7. Neuronal circuits may be protected by the TNF- α IP₃R signaling pathway: A proposed model

(1) Neuronal damage, from direct insults or the presence of toxic entities, can cause the dysfunctioning of upstream neurons in a neuronal circuit. (2) This damage can then lead to aberrant neurotransmitter release, which if unchecked, may lead to post-synaptic neuronal excitotoxicity. (3) The presence of proximal neuroinflammation can initiate the TNF- α IP₃R signaling pathway in C57BL/6 neurons. (4) Elevated IP₃R signaling leads to enhanced Ca²⁺-activated K⁺ channel function and hyper-polarization of the C57BL/6 neurons. (5) Neurotransmitter release is reduced, leading to the protection of downstream neurons (6). (7) In 3xTg-AD neurons, TNF-R expression is reduced through altered proteolytic processing by the gamma-secretase complex, thereby ablating TNF- α driven elevation of IP₃R-mediated Ca²⁺ release. (8) This may, in turn, lead to the further transmission of the excitotoxic signal eventually result in the death of downstream neurons and the spread of CNS damage (9).