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Cognitive Impairment in Humanized APPxPS1 Mice is Linked to $A\beta_{1-42}$ and NOX Activation

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Abstract

Cognitive impairment in Alzheimer's disease (AD) is strongly associated with both extensive deposition of amyloid β peptides and oxidative stress, but the exact role of these indices in the development of dementia is not clear. This study was designed to determine the relationship between cognitive impairment, activation of the free radical producing enzyme NADPH oxidase (NOX), and progressive changes in Aβ deposition and solubility in humanized APP × PS1 knock-in mice of increasing age. Data show that cognitive performance and expression of key synaptic proteins was progressively decreased in aging APP × PS1 mice. Likewise, NOX activity and expression of the specific NOX subunit NOX4 was significantly increased in APP × PS1 mice in an age-dependent manner, and NOX activity and cognitive impairment shared a significant linear relationship. Data further show that age-dependent increases in $A\beta_{1-42}$ had a significant linear relationship with both NOX activity and cognitive performance in APP × PS1 knock-in mice. Collectively, these data show that NOX expression and activity are significantly upregulated with age in this humanized model of Aβ pathogenesis, and suggest that NOX-associated redox pathways are intimately linked to both the loss of cognitive function and the deposition of $A\beta_{1-42}$.

Keywords

Aging; Dementia; Oxidative stress; Redox signaling pathways; Synapse loss

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INTRODUCTION

Alzheimer's disease (AD) is characterized by a progressive and irreversible loss of cognitive function and is the most common dementing disorder of the elderly (Jalbert et al., 2008). As existing treatments unfortunately have only limited efficacy in slowing clinical decline, there has been concerted effort shared by basic, clinical, and epidemiological researchers to map the etiology of this disease. An important and primary pathological feature of AD is the progressive accumulation of amyloid β-protein (Aβ) into neuritic plaques (Jellinger, 2002), (Selkoe, 2001), (Vinters and Farag, 2003), (Walsh and Selkoe, 2004), and the progression of cognitive decline is associated with accumulation of Aβ peptide, particularly $A\beta_{1-42}$, and alterations in Aβ solubility (Johnson, 2000), (Murphy et al., 2007). AD brains are also typified by ample pathological evidence of oxidative stress in affected regions, including the cerebral cortex and hippocampal formation (Markesbery and Lovell, 1998), (Aksenov et al., 2001), (Wang et al., 2006). Markers of oxidative stress are elevated in mild cognitive impairment (MCI), which may be the earliest stage of clinical dementia (Keller et al., 2005), (Williams et al., 2006), (Butterfield et al., 2006), (Mosconi et al., 2008). While collectively these data are consistent with the hypothesis that free radical production might drive the initiation of cognitive dysfunction, the nature of the relationship between free radical production and the pathogenesis of Aβ is still not fully understood.

Free radicals are produced in mammalian cells as secondary by-products by many different systems, including mitochondrial electron transport, xanthine oxidase, cyclooxgenases, and monoamine oxidases (reviewed in (Droge, 2002), (Reddy, 2006)). However, the enzyme NADPH oxidase (NOX) is noteworthy as it is dedicated to the specific and deliberate production of free radicals. Although NOX was first described in phagocytic cells such as microglia, it is now well established that NOX subunits are also expressed in neurons and astrocytes (Noh and Koh, 2000), (Kim et al., 2005). Indeed, experimental evidence points to a role for NOX in neuronal physiology, particularly in functions relating to hippocampal electrophysiology (Kishida et al., 2005), (Tejada-Simon et al., 2005). These data suggest that NOX is very well situated to participate in perturbations to cognitive function, and indeed, many reports have proposed that NOX may be involved in AD pathogenesis (reviewed in (Wilkinson and Landreth, 2006), (Block, 2008)). For example, early studies revealed the ability of Aβ to activate NOX and increase superoxide production (Meda et al., 1995), (Bruce et al., 1996), (Thomas et al., 1996), (Bianca et al., 1999), (Jana and Pahan, 2004), (Niikura et al., 2004), suggesting that NOX may be involved in Aβ-induced neuronal injury. Furthermore, published reports show increased NOX activity in AD brains (Shimohama et al., 2000) and (Ansari and Scheff, 2011), and also in MCI brains (Bruce-Keller et al., 2010) . Finally, genetic deletion of specific NOX subunits was recently shown to attenuate neurovascular dysfunction and cognitive decline in transgenic mice overexpressing the Swedish mutation of the human amyloid precursor protein (Park et al., 2008).

As there is ample support indicating that NOX could participate in AD, this study was undertaken to delineate the profile of NOX enzymatic activity and subunit expression in humanized APP × PS1 knock-in mice, which are a second generation, physiologically appropriate mouse model of Aβ pathogenesis. The use of knock-in technology, rather than a transgenic overexpression system, preserves the physiologically appropriate regulation of APP expression by endogenous promoters. Thus, data related to Aβ levels in these particular mice are not confounded by an artificial promoter system in unknown loci that drives supraphysiologic levels of APP expression. Indeed, previous reports from our group show that this model of Aβ pathogenesis is associated with progressive changes in Aβ solubility and deposition that mirror key changes in human AD (Flood et al., 2002), (Murphy et al., 2007). Furthermore, this model recapitulates the development of both diffuse and neuritic

plaques (Murphy et al., 2007), which is key to modeling AD pathology (Murphy et al., 2007). Finally, published reports document progressive and age-related increases in markers of oxidative stress in these mice (Abdul et al., 2008), (Huang et al., 2010). Thus, these studies were designed to specifically evaluate the exact relationship between Aβ, NOX activation, and cognitive decline. To this end, APP × PS1 and wild type (WT) mice were examined for cognitive impairment, and cognitive function was analyzed in relation to NOX activation and to parameters of Aβ deposition and solubility. Studies were conducted in young (4-6 month-old) mice, in which neuritic plaques are not detected; and old (16-19 month-old) mice, in which neuritic plaques are present predominantly in the frontal and parietal cortex and (to a lesser degree) in the hippocampus (Murphy et al., 2007).

MATERIALS AND METHODS

APP × PS1 knock-in mice

The Institutional Animal Care and Use Committee at the Pennington Center approved all experimental protocols, which were compliant with NIH guidelines on the use of experimental animals. All mice were housed in standard caging with 12:12 light: dark cycle, and had *ad libitum* access to food and water throughout the study. Young (age 4-6 months) and aged (16-19 months) mice were evaluated in this study, and data were compiled from 2 complete but separate cohorts of mice, for a total of 12-20 animals in each group.

This study employed $APP^{NLh/NLh} \times PS1^{P264L/P246L}$ mutant mice obtained from Cephalon, Inc. This second generation, knock-in model of Aβ pathology is based on Cre-lox knock-in technology, and expression of both genes is driven by their endogenous promoters and genes are not overexpressed (Anantharaman et al., 2006), (Reaume et al., 1996), (Siman et al., 2000), (Flood et al., 2002), (Chang et al., 2006). APP × PS1 mice are maintained on a CD-1/129 background, and wild type (WT) mice obtained from heterozygous APP × PS1 pairings were used as controls. The present study used only male mice.

Behavioral analysis of cognitive performance: Stone T-maze

Procedural memory was tested behaviorally in mice using the segmented Stone T-maze as described in previous reports (Pistell et al., 2010),(Pistell and Ingram, 2010), (Pistell et al., 2010). Briefly, performance in this maze requires the mouse to learn the correct sequence of 13 consecutive left and right turns to reach the goal box and successfully escape the maze. Mice are motivated to escape because they are required to wade (not swim) through the maze, as the apparatus is maintained in a tray of water (21-23°C) filled to a level (1.5 cm) that allows the mice to keep their head out of the water while maintaining contact with the floor, but the height of the maze prevents rearing. The day before acquisition testing, all animals were pre-trained to escape the water using a straight runway constructed of acrylic with opaque sides. For acquisition testing, mice were given 15 sequential trials in the T-maze in a single day. Mice were tested in groups of 8-10, and were run through such that the first trial was completed by all mice before proceeding to the second trial, insuring that each mouse had sufficient rest between trials to prevent any potential effects of fatigue. For the purpose of data analysis and presentation, maze acquisition data was collapsed and averaged into 5 separate “blocks” of 3 trials each. To measure memory retention, mice were given an additional 5 sequential trials in the maze 7 days after acquisition training. All trials were recorded using video tracking software (Viewpoint Lifesciences, Inc), and the numbers of errors committed were recorded and used as the primary measure of learning because it is unbiased by potential confounds resulting from differences in motor function.

Measures of synaptic marker and NOX subunit expression

Brain samples taken from the frontal cortex were homogenized in a Tris-buffered saline (pH 7.4) lysis buffer containing 0.1% Triton X-100, 5 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich). Samples were then denatured in SDS, and equivalent amounts of protein were electrophoretically separated in polyacrylamide gels and blotted onto nitrocellulose. Blots were processed using the following primary antisera: anti-p47phox (1:500, Millipore); anti-NOX2 (1:500, Santa Cruz Biotenchology, Inc.), anti-NOX4 (1:1000, Novus Biologicals LLC), anti-synapsin 1 (1:2000, Abcam) anti-phospho(S553)-synapsin 1 (1:10000), anti-synapse associated protein 97(1:3000, Thermo Fisher Scientific), and anti-tubulin (1:100, Wako Chemicals USA, Inc.). After incubation with primary antibodies, blots were washed and exposed to horseradish peroxidase-conjugated secondary antibodies, and visualized using a chemiluminescence system (Amersham Biosciences). Blot images were scanned and densitometrically analyzed for quantification. To ensure accurate quantification across multiple blots, samples from APP × PS1 and WT mice of a given age were included in each blot. Data were calculated as a ratio of expression over tubulin expression, which was included as an internal loading control. Protein expression in APP × PS1 mice was then calculated and presented as percent expression in WT mice of the same age so that multiple blots could be accurately reconciled.

Measures of NOX activity

Frozen samples taken from the frontal cortex of mice were homogenized in protease inhibitor-containing buffer at 4°C, and then subjected to differential centrifugation to isolate membranes. Membrane samples (exactly 35 μg total protein) were incubated with 5 μM lucigenin and 100 μM NADPH, and NOX activity was measured immediately by documenting the light produced by each sample at 37°C. Light emission was recorded from each sample in 10 second intervals for exactly 3 minutes. In agreement with published data on this assay (Li et al., 1998), (Janiszewski et al., 2002), initial optimization of this assay revealed that NADPH-driven luminescence was nearly completely prevented (>95% inhibition) by the addition of either diphenyleneiodonium (DPI), which selectively inhibits flavonoid-containing enzymes by irreversibly binding flavin (O'Donnell et al., 1993), or by superoxide dismutase, a catalytic scavenger of superoxide radicals (Fridovich, 1975). Thus, the specific role of NOX in the measured luminescence was determined by subtracting the background level of luminescence for each sample (generated by the inclusion of 1 μM DPI within the sample), and NOX activity is presented as average luminescent counts per minute (CPM) per microgram protein.

Analysis of Aβ solubility

Aβ solubility was measured using a well characterized three-step extraction procedure that uses sequentially more denaturing conditions to serially extract Aβ that is progressively more insoluble, and is followed by measurement of the amount of Aβ using a two-site (sandwich) ELISA. Details of this procedure, and the antibodies used, have been published (Beckett et al., 2010), (McGowan et al., 2005), (Murphy et al., 2007), but briefly, tissue was polytron homogenized in ice-cold PBS with a complete protease inhibitor cocktail (Amresco). The supernatant was collected following centrifugation at 20,000*xg* for 30 min at 4 °C. The pellet was re-extracted by brief sonication (10 × 0.5 s microtip pulses at 100 W; Fisher Sonic Dismembrator, Model 500) in 2% SDS with complete protease inhibitor cocktail, centrifuged, and the supernatant again collected. The remaining pellet was finally extracted by sonication (as above) in 70% formic acid (FA), and centrifuged at 20,000xg for 1 h at 4 °C. Sample extracts were stored frozen at − 80 °C until time of ELISA assay.

The sandwich ELISA was conducted using antibodies Ab42.5 (human sequence Aβ1–16) for $A\beta_{1\text{-}40}$ capture, and biotinylated 13.1.1 (end specific for $A\beta_{1\text{-}40}$) for detection.

Measurement of $A\beta_{1-42}$ was done with antibodies 2.1.3 (end specific for $A\beta_{1-42}$) for capture and biotinylated 4G8 (human sequence Aβ17-24, Covance) for detection. All standards and samples were run at least in duplicate. Plates (Immulon 4HBX) were coated with 0.5 μg/well of antibody in PBS, and blocked with Synblock (Serotec) overnight. This is a well characterized system, with a lower limit of detection of approximately 5 pM and a linear range of about two orders of magnitude (20–2000 pM). Following development with TMB reagent, plates were stopped with 6% *o*-phosphoric acid and read at 450 nm using a BioTek multiwell plate reader. Total levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ were determined by calculating the sum of levels in each fraction (PBS, SDS, and formic acid).

Statistical analyses

All data are shown as mean ± standard error of measurement. Stone maze performance was analyzed with 2-way repeated measures analyses of variance (ANOVA) to determine main effects of trail block and treatment, followed by Bonferroni post-hoc comparisons to determine differences between APP × PS1 and WT groups. NOX activity was also analyzed with 2-way ANOVA to determine main effects of genotype and age, followed by Bonferroni post-hoc comparisons to determine differences between APP × PS1 and WT mice of each age. Data generated from Western blot were analyzed by 2-tailed, unpaired t-tests to determine specific differences between genotypes within individual age groups. Linear regression analyses were used to determine statistical relationships of NOX activity and Aβ levels with cognitive decline. Statistical significance for all analyses was accepted at $p < 0.05$, and *, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

RESULTS

Cognitive function in young and aged APP × PS1 and WT mice

Previous behavioral investigations of cognitive function in APP × PS1 knock-in mice employed the Morris water maze and the 6-arm water maze tasks of spatial memory (Chang et al., 2006). This study did not reveal a working memory deficit in APP × PS1 mice, although APP × PS1 mice did show impaired memory “flexibility”, i.e., the ability to learn the location of a new platform each day without perseverating on the previous location (Chang et al., 2006). However, it is possible that deficits in working memory may have been masked in the water-based assays, as it is generally appreciated that mice perform uniformly poorly in learning tasks that require swimming (Whishaw and Tomie, 1996). Thus, we sought to assess cognitive function in APP × PS1 mice using the Stone T-maze maze, which is a task of procedural learning and memory that is very well suited for use in mice as it does not require the animals to swim, and is not confounded by changes in motor impairment, feeding behavior, or nociception (Pistell and Ingram, 2010), (Pistell et al., 2010), (Pistell et al., 2010). This task, which is described in detail in Methods, was developed following the success of this maze in assessing age-related cognitive deficits in rats (Ingram, 1988), (Spangler et al., 1989), (Markowska et al., 1989). In addition to documenting age-related cognitive decline, this task has been successfully used in both rats and mice to document the detrimental effects of cholinergic antagonists (Spangler et al., 1989), (Bratt et al., 1994) , cerebral ischemia (Caldwell et al., 1997), and diabetes/obesity (Pistell et al., 2010), (Morrison et al., 2010), (Stranahan et al., 2008) on cognitive function. Furthermore, studies in rats in which both Stone maze and Morris water maze tasks were employed demonstrated that errors made during Stone maze training mirrored latency data obtained using the Morris water maze (Lee et al., 2006), (Markowska et al., 1989). Young (age 4-6 months) and aged (16-19 months) mice were thus evaluated for both acquisition and retention in the Stone maze as described in Methods, and data show that aged APP × PS1 knock-in mice were significantly impaired in maze acquisition (Fig 1A). Specifically, ANOVA on errors committed by 16-19 month-old mice revealed significant main effects of trial block ($F_{(4, 80)}$

= 54.2, $p < 0.0001$) and genotype ($F_{(1,80)} = 24.8$, $p < 0.0001$). Post-hoc analyses revealed that APP × PS1 mice committed significantly more errors in the trial blocks 1-3, 4-6, and 7-9 as compared to WT mice (Fig. 1A). Maze acquisition was also impaired in young APP × PS1 knock-in mice, but to a lesser degree (Fig. 1B). Specifically, ANOVA on errors committed by 4-6 month-old mice revealed significant main effects of trial block ($F_{(4, 80)} = 54.8$, $p < 0.0001$) and genotype ($F_{(1,80)} = 4.6$, $p < 0.05$), but post-hoc analyses did not indicate significant differences in performance in individual trial blocks (Fig. 1B).

Maze retention was evaluated 7 days following acquisition as described in Methods, and data show a severe impairment in memory retention in aged APP × PS1 knock-in mice (Fig 1C). 2-way ANOVA on retention errors committed by 16-19 month-old mice revealed a significant main effect of trial number ($F_{(4, 80)} = 3.4$, $p < 0.05$) and genotype ($F_{(1,80)} = 27.3$, $p < 0.0001$). Post-hoc analyses revealed that aged APP × PS1 mice committed significantly more errors in the first 2 retention trials as compared to WT mice (Fig. 1C). Retention was also impaired in young APP × PS1 knock-in mice, (Fig. 1D). ANOVA on errors committed by 4-6 month-old mice revealed significant main effects of trial number ($F_{(4, 80)} = 5.5$, $p < 0.001$) and genotype ($F_{(1,80)} = 15.4$, $p < 0.001$), and post-hoc analyses revealed a significant increase in errors committed by APP × PS1 mice in the second retention trial (Fig. 1D).

Synaptic marker expression in young and aged APP × PS1 and WT mice

Experiments were next designed to determine if the observed decreases in memory function were associated with altered expression of key synaptic markers. To this end, expression of the post-synaptic protein synapse associated protein 97 (SAP97) and total and phosphorylated forms of the pre-synaptic protein synapsin 1 (SYN1) were quantified in tissue homogenates prepared from the frontal cortex by Western blot as described in Methods. These specific markers were chosen as studies have shown that these proteins reflect most faithfully the number of synapses as determined by EM-based synapse counts (S.W. Scheff, personal communication). Each age (4-6 and 16-19 months) was measured separately by Western blot, and individual samples were normalized first to tubulin and then to WT to reconcile multiple blots, as described in Methods. Thus, for each age, protein expression values from APP ×PS1 mice were compared to values from WT mice by t-test. Quantification of SAP97 expression revealed that levels were significantly decreased in APP × PS1 mice (Fig. 2A). Specifically, 2-tailed, unpaired t-tests indicated significant decreases in SAP97 expression in both 4-6 month old ($t_{(38)} = 2.07$, $p = 0.045$) and 16-19 month-old APP × PS1 mice ($t_{(36)} = 3.08$, $p = 0.0039$) as compared to WT mice of the same age (Fig. 2A). Conversely, total SYN1 expression in frontal cortex was not altered in APP × PS1 mice as compared to WT mice of either age (Fig. 2B).

However, levels of phosphorylated SYN1 were affected in APP × PS1 mice (Fig. 2C and 2D). Specifically, 2-tailed, unpaired t-tests revealed that while the trend towards lower levels in 4-6 month-old APP × PS1 mice did not reach statistical significance,16-19 month-old mice had significantly reduced phosphorylated SYN1 expression ($t_{(36)} = 3.89$, $p = 0.0004$) in the cortex as compared to WT mice (Fig. 2C).

NOX activity in young and aged APP × PS1 and WT mice

The pro-oxidant and pro-inflammatory enzyme NOX has been proposed as an important potential mediator of AD pathology (Wilkinson and Landreth, 2006), (Block, 2008). While reports from our group and others have shown increased NOX activity in AD and MCI brains (Shimohama et al., 2000), (Bruce-Keller et al., 2010), (Ansari and Scheff, 2011), and in transgenic animal models (Jana and Pahan, 2004), (Niikura et al., 2004), (Park et al., 2008), the profile of NOX enzymatic activity in this humanized, knock-in model of AD has not been established. Thus, NOX activity was determined in frontal cortex of both young

and aged WT and APP × PS1 mice as described in Methods. Data show that NOX activity is increased in APP × PS1 mice, particularly in 16-19 month-old mice (Fig. 3). Specifically, 2-way ANOVA of NOX activity in young and old APP × PS1 and WT mice revealed significant main effects of both age ($F_{(1,72)} = 5.84$, $p = 0.018$) and genotype ($F_{(1,72)} = 18.2$, $p < 0.0001$) on NOX activity, and a significant interaction of age x genotype ($F_{(1,72)} = 6.08$, $p = 0.016$). Bonferroni post-hoc analyses revealed that NOX activity in 16-19 month-old APP × PS1 mice was significantly elevated compared to activity in WT mice of the same age (Fig. 3), but no differences were noted in 4-6 month-old mice.

NOX subunit expression in young and aged APP × PS1 and WT mice

Experiments next determined if the observed increases in NOX activity were associated with enhanced expression of key NOX subunits. To this end, the expression profile of the regulatory subunit p47phox, the catalytic subunit NOX2 (gp91phox), and the NOX2 homolog NOX4 in the frontal cortex of young and aged WT and APP × PS1 mice was measured by Western blot as described in Methods. Evaluation of p47phox expression in frontal cortex did not indicate changes in APP × PS1 mice as compared to WT mice of either age (Fig. 4A). Likewise, expression of NOX2 was not significantly altered in APP × PS1 mice of either age (Fig. 4B). Conversely, levels of NOX4 were significantly increased in APP × PS1 mice (Fig. 4C and 4D). Specifically, 2-tailed, unpaired t-tests revealed significant increases in NOX4 expression in 16-19 month-old APP × PS1 mice ($t_{(20)} = 3.76$, $p = 0.0012$) as compared to WT mice, but not in 4-6 month-old mice (Fig. 4C). Levels of the alternate NOX2 homologs NOX1 and NOX3 were also evaluated in young and old WT and APP × PS1 mice, but the expression of these proteins in the brain was beneath the level of detection by Western blot (data not shown).

Relationship of NOX activity and behavioral performance in young and aged APP × PS1 and WT mice

Regression analyses were next undertaken to determine the statistical relationship between NOX activity and cognitive function. Specifically, numerical values for NOX activity were correlated against errors accrued by the same individual mice in acquisition trials 4-6 in young and aged APP × PS1 and WT mice. Data analyses show a strong and statistically significant association between overall NOX activity in the frontal cortex and acquisition errors in 16-19 month-old mice ($r^2 = 0.4105$, $p = 0.0042$; Fig. 5A). The relationship between NOX activity in the frontal cortex and acquisition errors in 4-6 month-old mice was also significant ($r^2 = 0.2912$, $p = 0.0208$; Fig. 5B). NOX activity also showed a significant correlation with errors in retention trials 1-3 in both aged and young mice (data not shown).

Progressive changes in Aβ levels and solubility in APP × PS1 mice

Previous reports from our group show that this humanized model of Aβ pathogenesis recapitulates the development of both diffuse and neuritic plaques, and is furthermore associated with progressive changes in Aβ solubility and deposition that mirror the progressive solubility changes observed in human AD (Flood et al., 2002), (Murphy et al., 2007). To determine how the cognitive abnormalities noted in these mice related to physiologic changes in Aβ indices, alterations in Aβ levels and solubility were evaluated in young and aged APP × PS1 mice. Total levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ were evaluated as described in Methods, and showed dramatic increases in 16-19 month-old mice as compared to 4-6 month-old mice (Table 1). Furthermore, $Aβ_{1-40}$ was increased within the PBS, SDS, and formic acid pools in 16-19 month-old mice relative to young mice (Table 1). Indeed, formic acid extractable $Aβ_{1-40}$ could not be detected in young mice, but was abundant in the aged group (Table 1). Likewise, neither SDS extractable $Aβ_{1-42}$ nor formic acid extractable $Aβ_{1-42}$ could be detected in young mice, but $Aβ_{1-42}$ partition into these increasingly insoluble fractions was apparent in the aged group (Table 1). Levels of $Aβ_{1-40}$ and $Aβ_{1-42}$ in

WT mice of the same genetic background were not elevated significantly above background levels (data not shown).

Relationship of Aβ to NOX activity and behavioral performance in APP × PS1 mice

To determine how these Aβ indices relate to the cognitive and physiologic abnormalities noted in these mice, regression analyses were next undertaken to determine the statistical relationship between Aβ levels in specific pools to NOX activity and cognitive function. Specifically, numerical values for total $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, and for $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in PBS, SDS, and formic acid extractable fractions were correlated against NOX activity in the frontal cortex and against acquisition errors in the same individual young and aged APP × PS1 mice. Data analyses show that for 4-6 month-old APP × PS1 mice, no correlation could be found for any pool of $A\beta_{1\text{-}40}$ or $A\beta_{1\text{-}42}$ (total, PBS, or SDS soluble) to either acquisition errors or NOX activity (data not shown). Likewise, for 16-19 month-old APP × PS1 mice, no correlation could be found for total $A\beta_{1\text{-}40}$, or for PBS-, SDS-, or formic acid soluble $A\beta_{1\text{-}40}$ with overall NOX activity or acquisition errors (data not shown). However, there was significant correlation between $A\beta_{1\text{-}42}$ and acquisition errors in 16-19 month-old APP × PS1 mice (Fig. 6). Specifically, acquisition errors in 16-19 month-old APP × PS1 mice correlated significantly with both SDS-soluble $A\beta_{1\text{-}42}$ ($r^2 = 0.7514$, $p = 0.0012$; Fig. 6A) and total $A\beta_{1\text{-}42}$ ($r^2 = 0.7614$, $p = 0.001$; Fig. 6B). There was not a significant linear relationship between PBS-soluble $A\beta_{1\text{-}42}$ and acquisition errors ($r^2 = 0.0088$, $p = 0.7971$; not shown), but levels of formic acid-soluble $A\beta_{1\text{-}42}$ did show a modest but significant correlation with acquisition errors in16-19 month-old APP × PS1 mice ($r^2 = 0.4018$, $p = 0.049$; not shown). The statistical relationship between $A\beta_{1\text{-}42}$ and NOX activity in the frontal cortex of 16-19 month-old APP × PS1 mice was also significant, although not as strong as for acquisition errors (Fig. 6). NOX activity correlated significantly with SDS-soluble $A\beta_{1\text{-}42}$ ($r^2 = 0.4215$, $p = 0.0306$; Fig. 6C) and total $A\beta_{1\text{-}42}$ ($r^2 = 0.4975$, $p = 0.0153$; Fig. 6D). However, there was no correlation between PBS-soluble $A\beta_{1\text{-}42}$ and NOX activity ($r^2 = 0.0409$, $p = 0.5506$; not shown), nor between levels of formic acid-soluble $A\beta_{1\text{-}42}$ and NOX activity ($r^2 = 0.1249$, $p = 0.2865$; not shown) in 16-19 month-old APP × PS1 mice. These data are generally comparable to the degree of correlation found in published reports employing similar linear regression techniques in conjunction with Aβ analyses. For example, the correlation demonstrated between fronto-cortical fibrillar Aβ accumulation and dementia as measured by MMSE in humans is $r = .72$ / $r^2 = .518$ (Sarsoza et al., 2009). Additionally, the reported correlation coefficients between the number of synaptophysin-immunoreactive terminals and age in wild-type human amyloid protein precursor transgenic mice ($r = 0.53$ / $r^2 = .2809$; Mucke et al., 2000) and between memory deficit and neurogenesis in 3xTgAD mice ($r = .80$ / $r^2 = .64$; Wang et al., 2010) are also comparable to data in this manuscript.

DISCUSSION

This study uses a second generation mouse model of AD to evaluate the role of NOX in amyloid pathogenesis. Specifically, using APP × PS1 knock-in mice, experiments were designed to document changes in cognitive function and NOX activity, and to further evaluate these indices in relation to changes in Aβ. Data show that NOX activity is significantly increased in aged APP × PS1 mice, and furthermore shares a significant linear relationship with deficits in cognitive function. Data also show that while p47phox and NOX2 (gp91phox) expression are not altered in APP × PS1 mice, expression of the NOX2 homolog NOX4 is significantly increased in aged APP × PS1 mice. Finally, data further show that both NOX activity and cognitive function show a significant linear relationship specifically with levels of $A\beta_{1\text{-}42}$, but not $A\beta_{1\text{-}40}$. Overall, these data are in general agreement with published studies suggesting a role for NOX in the pathogenesis of AD (Ansari and Scheff, 2011), (Park et al., 2008), (Bruce-Keller et al., 2010), but extend these

studies by demonstrating the intimate link between NOX activity and both disruptions in cognitive function and the deposition of specific forms of Aβ. The knock-in mice are powerful tool with which to study Aβ pathology, and indeed, our previous reports show that this model of Aβ pathogenesis is associated several key phenomena that are also observed in AD brain, including progressive changes in Aβ solubility and the development of both diffuse and neuritic plaques. Furthermore, data obtained from these mice are not hindered by confounds caused by an artificial promoter system, ectopic expression, or supraphysiologic levels of APP expression. Thus, the combination of this unique animal model of Aβ pathology with stringent behavioral and biochemical analyses has revealed a potentially critically important role for NOX activation in Aβ pathogenesis. While cause and effect relationships remain to be fully resolved, these data strongly suggest that NOX could be a key factor linking SDS-soluble $A\beta_{1\text{-}42}$ to disruptions in synaptic/cognitive function.

While the etiology of AD-related cognitive decline is still not understood, advanced age is the strongest risk factor for AD and the free radical theory of aging (Harman, 1956) may have particular relevance to the development of AD. This oxidative stress-based theory describes a redox imbalance, whereby the production of free radicals overtakes endogenous anti-oxidant capacity, leading to oxidative damage to critical cellular elements (Harman, 1993). Indeed, oxidative stress has been repeatedly implicated in both brain aging and AD (Morrison et al., 2010), (Liu et al., 2003), (Joseph et al., 1998), (Butterfield et al., 2001), (Markesbery and Lovell, 1998), (Stadtman and Levine, 2003), but the primary source of AD-related oxidative stress has not been identified. There is strong evidence supporting mitochondrial dysfunction in AD, and the “mitochondrial cascade hypothesis” posits that age-related increases in basal mitochondrial ROS production lead to neuronal dysfunction and pathological hallmarks of AD (Swerdlow and Khan, 2004). Data in this manuscript suggest that increased NOX might participate with mitochondria in AD-associated oxidative stress and cognitive decline, which is in keeping with many reports implicating NOX in AD (Wilkinson and Landreth, 2006), (Park et al., 2008), (Park et al., 2005). It is also important to note that reactive oxygen species (ROS) are important signaling molecules involved in synaptic plasticity and memory formation, and that NOX in particular is a key regulator of ROS generation in synaptic plasticity and memory formation (reviewed in (Kishida and Klann, 2007)). Thus, aberrant NOX activation could significantly perturb cognitive function even in the absence of overt oxidative stress. In support of this scenario, experimental data indicate a delicate balance of ROS required for signaling, with either too little or too much ROS resulting in impairments in long term potentiation (LTP) and memory (Knapp and Klann, 2002). In further reference to aging, data suggest the presence of an age-related shift in the role of ROS signaling in memory formation (Knapp and Klann, 2002), (Kamsler and Segal, 2004), (Hu et al., 2006), as overexpression of antioxidant enzymes impairs LTP in young mice, but preserves LTP in aged mice (Hu et al., 2006), (Kamsler and Segal, 2004). Likewise, long-term application of antioxidant enzyme mimetics can reverse hippocampus-dependent learning deficits in aged mice (Liu et al., 2003). While the exact biochemical means whereby NOX modulates physiologic signaling are still under investigation, current theories focus on reversible thiol oxidation of cysteine residues, a chemical alteration often utilized by ROS to impact signaling without causing cytotoxicity (Parasassi et al., 2010), and protein phosphatases are well known to be inhibited by reversible cysteine oxidation (Denu and Tanner, 1998). Finally, it should be noted that the detrimental actions of NOX appear to be most strongly associated with age-related chronic disease processes, including Parkinson’s disease and atherosclerosis in addition to AD (reviewed in (Lambeth, 2007)). Indeed, a model of “antagonistic pleiotropy” has been proposed to explain the potentially dual role of NOX with age, in which the physiologic production of ROS garners an advantage in early life but sustained or aberrant activation of NOX culminates in harmful effects with age later in life (Lambeth, 2007), supporting the potential detrimental role of NOX in the pathogenesis of AD.

NOX, which is uniquely dedicated to the deliberate and specific production of reactive oxygen species, consists of membrane (NOX2, also called gp91phox, and p22phox) and cytosolic (p47phox, p67phox, and p40phox) components (Babior, 1991), (DeLeo and Quinn, 1996). The membrane-integrated protein NOX2 is the catalytic core of the enzyme responsible for the electron transfer from NADPH to molecular oxygen for superoxide production. Interestingly, recent expansion of the genome database has led to identification of several novel NOX2 homologues which constitute the NOX family of oxidases. These proteins are distinct from the phagocyte NOX2 and have functions other than host defense (Lambeth, 2004), (Geiszt and Leto, 2004). The human genome contains 5 NOX members: NOX1 through NOX5, with NOX2 as gp91phox. Some of these novel oxidases have a fairly limited tissue expression (Krause, 2004), but both NOX1 and NOX4 are thought to be expressed in the CNS in addition to NOX2 (Lambeth, 2004), (Krause, 2004), (Sorce and Krause, 2009). Indeed, data in this report suggest that increased expression of NOX4 could contribute to the observed increases in NOX activity in aged APP × PS1 mice. While NOX activity is regulated post-translationally, and thus increased subunit expression is not necessary *per se* for increased enzyme activity, studies have shown that increased expression of either NOX2 or NOX4 is sufficient to increase NOX activity in transgenic mice (Anilkumar et al., 2008). Furthermore, the increased expression of NOX4 in human rennin/angiotensinogen chimeric transgenic mice is associated with both increased NOX activity and cognitive decline (Inaba et al., 2009). Thus, NOX4 may be a key participant in the increased NOX activity that has been repeatedly noted in the progression of AD (Ansari and Scheff, 2011), (Bruce-Keller et al., 2010). Indeed, the contribution of NOX4 to amyloid pathology may partially explain why administration of the widely used NOX-inhibitor apocynin, which has been shown to be protective in models of stroke and ALS (Harraz et al., 2008), (Tang et al., 2008), (Wang et al., 2006), was recently shown to be ineffective in preventing cognitive deficits, amyloid deposition, microgliosis and hyperphosphorylated tau in the Tg19959 mouse model (Dumont et al., 2011). Apocynin is thought to inhibit NOX by preventing the assembly of NOX subunits, particularly the translocation of p47phox (Stolk et al., 1994), which may not be a prerequisite for activation of NOX4, as evidence suggests that NOX4 is constitutively active and does not require the cytosolic subunits (Ray and Shah, 2005) (Martyn et al., 2006).

It is widely thought that AD begins as a malfunction of synapses, eventually leading to cognitive impairment and dementia. Indeed, extensive work on AD indicates that memory failure in early AD patients results from synaptic disruption without frank neuronal loss (reviewed in (Selkoe, 2002)), and this perturbation is attributed to the toxicity of the 42-aa variant of the amyloid β protein ($A\beta_{1\text{-}42}$). Furthermore, work in multiple AD models (Rowan et al., 2003) has shown that $A\beta_{1\text{-}42}$ in particular impairs synaptic plasticity in brain regions that are recognized early targets for AD. Thus, while the specific role that amyloid beta peptides (Aβ) play in causing AD is subject to controversy, it is clear that Aβ-containing senile plaques are associated with degenerating neurons, and that Aβ peptides are potently bioactive both *in vitro* and *in vivo*, and collective evidence suggests that AD may be mediated at least in part via the overproduction of Aβ (reviewed in (Selkoe and Schenk, 2003)). Thus, a significant finding in the present study is the significant linear association between both NOX activity and cognitive performance in individual mice with levels of $A\beta_{1\text{-}42}$. As published data convincingly demonstrate that Aβ can increase NOX activity in neurons and microglia (Meda et al., 1995), (Bruce et al., 1996), (Thomas et al., 1996), (Bianca et al., 1999), (Niikura et al., 2004), (Jana and Pahan, 2004), (Shelat et al., 2008), these data suggest that $A\beta_{1\text{-}42}$, particularly within increasingly insoluble SDS fractions, may disrupt synaptic and cognitive function via the induction of NOX.

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Highlights

- Cognitive performance and expression of key synaptic proteins is progressively decreased with age in APP × PS1 mice
- NOX activity and expression of the NOX4 is progressively increased with age in APP × PS1 mice
- Cognitive function shows a significant linear correlation with NOX activity and Aβ1-42

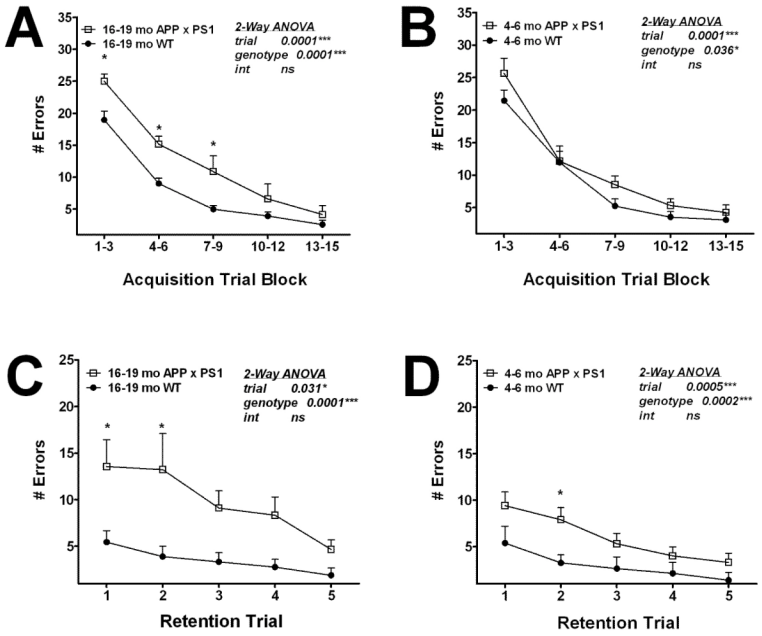

Figure 1. Age-related impairment in cognitive ability in APP × PS1 mice

Male APP × PS1 and WT mice were tested behaviorally in the Stone T-maze as described in Methods. **(A)** Procedural memory was evaluated in aged (16-19 month-old) APP × PS1 and WT mice by quantifying the number of errors committed over 15 trials of maze acquisition training. Data are means ± S.E.M. of average errors accrued over 3-trial blocks and were generated from 9-12 mice per group. Data were analyzed by 2-way ANOVA, and * indicates significant ($p<0.05$) increases in errors made by APP × PS1 mice. **(B)** Procedural memory in young (4-6 month-old) mice was measured by quantifying the number of errors committed over 15 trials of maze acquisition training. Data are means ± S.E.M. of average errors accrued over 3-trial blocks and were generated from 9-12 mice per group, and were analyzed by 2-way ANOVA. **(C)** Memory retention was tested in aged (16-19 month-old) APP × PS1 and WT mice by quantifying the number of errors committed over 5 additional trials in the maze 7 days following acquisition training. Data are means ± S.E.M. of average errors accrued over 5 individual trails, and were analyzed by 2-way ANOVA. * indicates significant ($p<0.05$) increases in errors made by APP × PS1 mice. **(D)** Memory retention in young (4-6 month-old) APP × PS1 and WT mice was measured by 5 additional trials in the maze 7 days following acquisition training. Data are means ± S.E.M. of average errors accrued over 5 individual trails, and were analyzed by 2-way ANOVA. * indicates significant ($p<0.05$) increases in errors made by APP × PS1 mice.

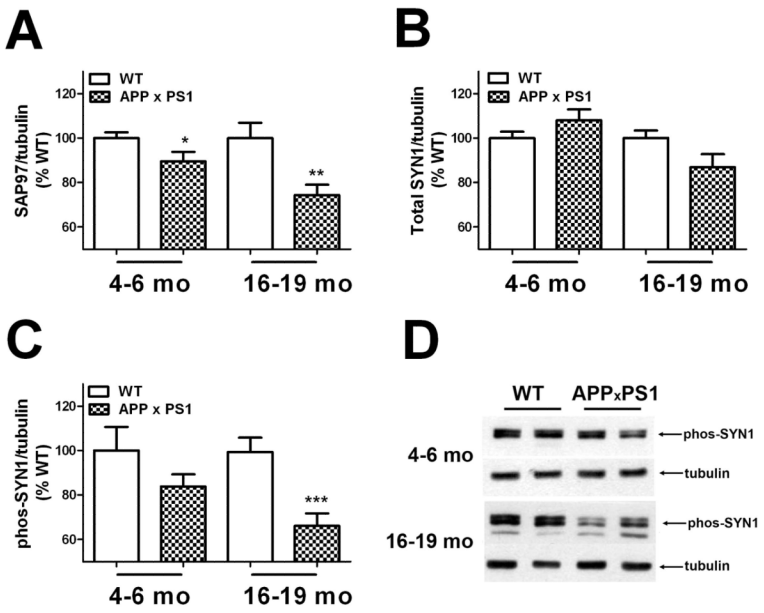

Figure 2. Age-related decreases in synaptic marker expression in APP × PS1 mice

(A) Expression of the post-synaptic protein synapse associated protein 97 (SAP97) was evaluated in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice by Western blot as described in Methods. For consistent quantification across multiple blots, individual samples were normalized first to tubulin and then to levels in WT mice, as described in Methods. Data are mean ± SEM SAP97 expression in 19-20 mice/group, and were analyzed by 2-tailed, unpaired t-tests. * and ** indicate significant ($p < 0.05$ and 0.01, respectively) decreases in SAP97 expression detected in young and aged APP × PS1 mice, respectively. **(B)** Expression of the pre-synaptic protein synapsin 1 (SYN1) was evaluated in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice as described in Methods. Data are mean ± SEM SYN1 expression in 19-20 mice/group. **(C)** Expression of phosphorylated synapsin 1 (phos-SYN1) was evaluated in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice as described in Methods. Data are mean ± SEM phos-SYN1 expression in 19-20 mice/group, and were analyzed by 2-tailed, unpaired t-tests. *** indicates significant ($p < 0.001$) decreases in phos-SYN1 expression in aged APP × PS1 mice as compared to aged WT mice. **(D)** Representative images depict the pattern of phos-SYN1 expression in young and aged APP × PS1 and WT mice.

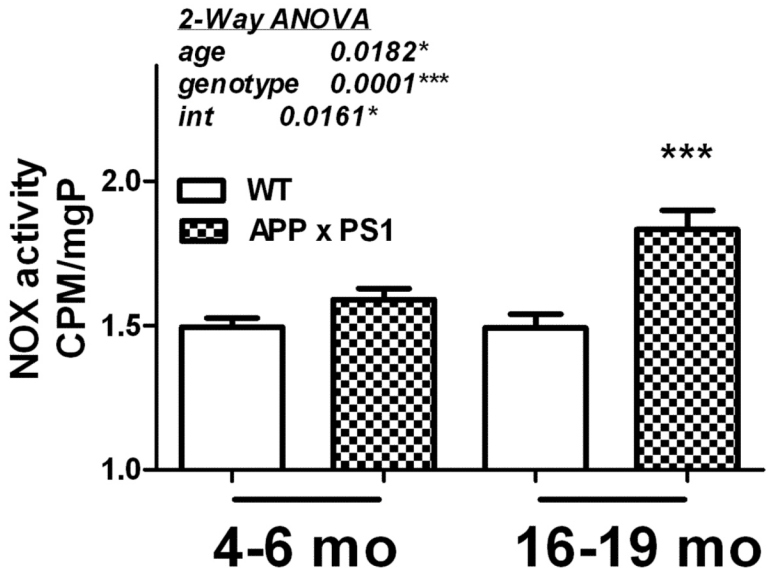

Figure 3. Age-related increases in NOX activity in APP × PS1 mice

NOX activity was measured in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice using lucigenin as described in Methods. Data were calculated as counts per minute (CPM) per microgram total protein, and are presented as mean ± SEM. 2-way ANOVA indicated an overall significant main effect of age and genotype on NOX activity, and also a significant interaction of age and genotype on NOX activity (text insert). *** indicates significant ($p < 0.001$) increases in NOX activity in aged APP × PS1 mice as compared to aged WT mice.

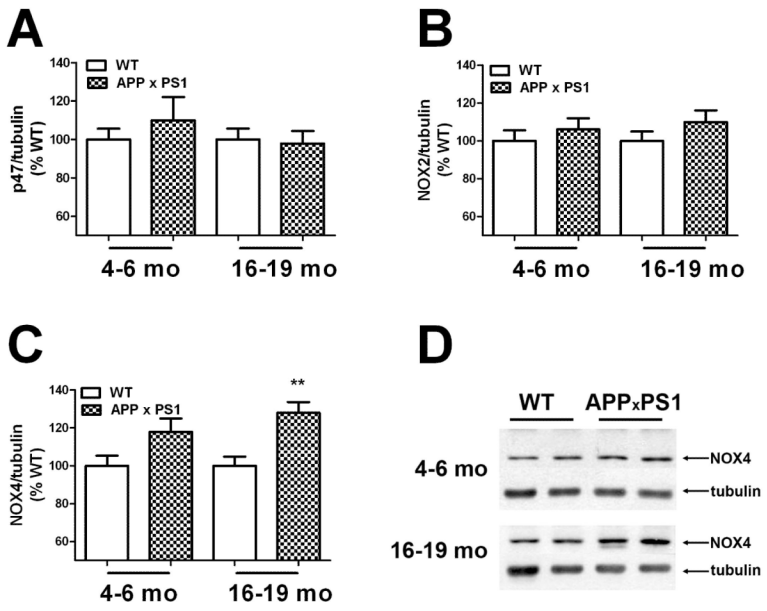

Figure 4. NOX subunit expression in young and aged WT and APP × PS1 mice

(A) Expression of the regulatory subunit p47phox (p47) was evaluated in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice by Western blot as described in Methods. For consistent quantification across multiple blots, individual samples were normalized first to tubulin and then to levels in WT mice, as described in Methods. Data are mean ± SEM p47expression in 19-20 mice/group, and were analyzed by 2-tailed, unpaired t-tests. **(B)** Expression of the catalytic subunit NOX2 was evaluated in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice as described in Methods. Data are mean ± SEM NOX2 expression in 19-20 mice/group. **(C)** Expression of NOX2 homolog NOX4 was evaluated in tissue homogenates prepared from the frontal cortex of young (4-6 month–old) and aged (16-19 month–old) APP × PS1 and WT mice as described in Methods. Data are mean ± SEM NOX4 expression in 19-20 mice/group, and were analyzed by 2-tailed, unpaired t-tests. ** indicates significant ($p < 0.01$) increases in NOX4 expression in aged APP × PS1 mice as compared to aged WT mice. **(D)** Representative images depict the pattern of NOX4 expression in young and aged APP × PS1 and WT mice.

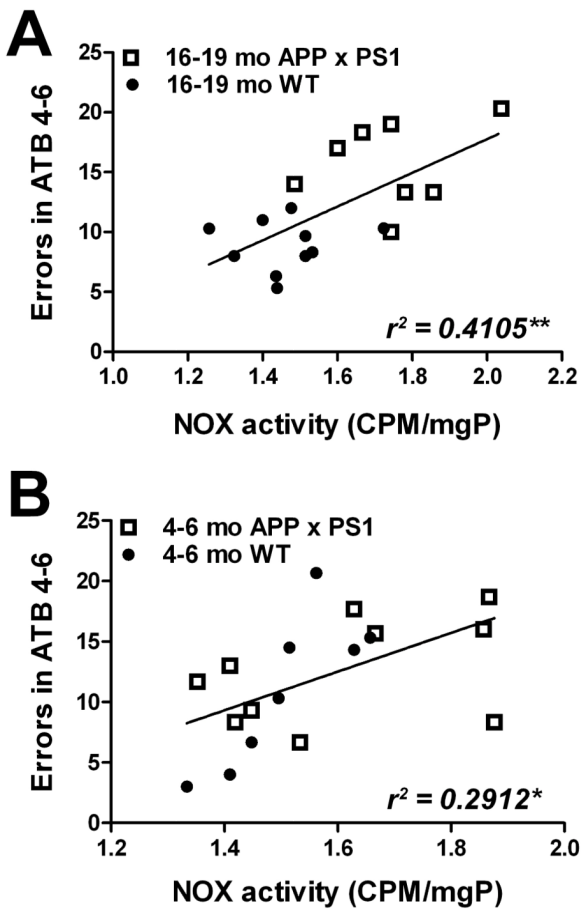

Figure 5. Linear relationship between maze performance and NOX activity in young and aged WT and APP × PS1 mice

(A) Scatter plots show the relationship between errors in acquisition trail block 4-6 (errors in ATB 4-6) and NOX activity in aged (16-19 month-old) APP × PS1 (open squares) and WT (closed circles) mice. Each point represents an individual subject, and the line depicts the degree of linear regression. **(B)** Scatter plots show the relationship between errors in acquisition trail block 4-6 (errors in ATB 4-6) and NOX activity in young (4-6 month-old) APP × PS1 (open squares) and WT (closed circles) mice. Each point represents an individual subject, and the line depicts the degree of linear regression.

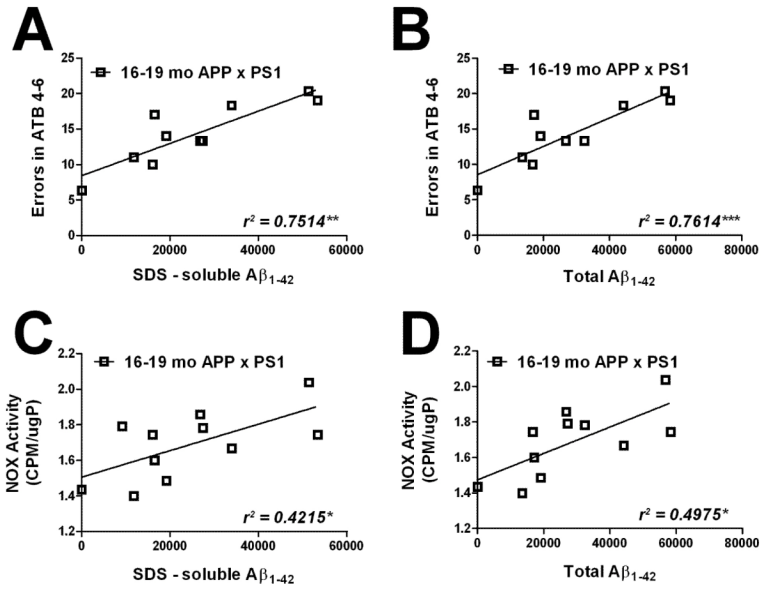

Figure 6. Linear relationship between $A\beta_{1\text{-}42}$ and both maze performance and NOX activity in aged APP × PS1 mice

(A) Scatter plots show the relationship between errors in acquisition trail block 4-6 (errors in ATB 4-6) and SDS-soluble $A\beta_{1\text{-}42}$ levels in 16-19 month-old APP × PS1 mice. Each point represents an individual subject, and the line depicts the degree of linear regression. **(B)** Scatter plots show the relationship between errors in acquisition trial block 4-6 (errors in ATB 4-6) and total $A\beta_{1\text{-}42}$ levels in 16-19 month-old APP × PS1 mice. Each point represents an individual subject, and the line depicts the degree of linear regression. **(C)** Scatter plots show the relationship between NOX activity and SDS-soluble $A\beta_{1\text{-}42}$ levels in 16-19 month-old APP × PS1 mice. Each point represents an individual subject, and the line depicts the degree of linear regression. **(D)** Scatter plots show the relationship between NOX activity and total $A\beta_{1\text{-}42}$ levels in 16-19 month-old APP × PS1 mice. Each point represents an individual subject, and the line depicts the degree of linear regression.

Table 1

Summary of Aβ levels and progressive changes in solubility in young (age 4-6 months) and aged (16-19 months) APP X PS1 mice

To quantify levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in increasingly insoluble fractions, Aβ proteins were examined by serial extraction from phosphate buffered saline (PBS), sodium dodecyl sulfate (SDS), and formic acid (FA) soluble fractions, followed by ELISA as described in Methods. Total levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were determined by summing levels detected in all fractions. Data are expressed as mean pmol/g tissue ± SEM with 10-12 mice per group. ND = not detected.

Aβ 1-40	**PBS**	**SDS**	**FA**	**Total**
4-6 months	86.7 ± 6.1	1,623.1 ± 74.6	*ND*	1,709.8 ± 73.0
16-19 months	153.5 ± 10.5	39,518.7 ± 5108.9	11,022.2 ± 2418.7	50,694.5 ± 4475.7
Aβ 1-42	PBS	SDS	FA	Total
4-6 months	22.7 ± 10.4	*ND*	*ND*	22.7 ± 10.4
16-19 months	16.7 ± 7.5	26,571.4 ± 4921.8	4,653.3 ± 1729.1	31,241.4 ± 4450.1