Aging and excitotoxic stress exacerbate neural circuit reorganization in amyloid precursor protein intracellular domain transgenic mice

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Abstract

The cleavage of amyloid precursor protein (APP) by presenilins simultaneously generates amyloid-β (Aβ) and APP intracellular Domain (AICD) peptides. Aβ plays a pivotal role in Alzheimer’s disease (AD) pathology and recently AICD was also shown to contribute to AD. Transgenic mice overexpressing AICD show age-dependent tau phosphorylation and aggregation, memory deficits, and neurodegeneration. Moreover, these mice show aberrant electrical activity and silent seizures beginning at 3–4 months of age. Here we show that AICD mice also displayed abnormal mossy fiber sprouting beginning about the same time and that this sprouting intensified as the animals aged. Expression of neuropeptide Y was increased in mossy fiber terminals in aged but not young AICD mice. Importantly, young AICD mice injected with kainic acid showed similar pathology to that observed in aged AICD mice. These data show that elevated levels of AICD render neurons hypersensitive to stress and induce hippocampal circuit reorganization, which can further exacerbate hyperexcitability. These results further demonstrate that AICD, in addition to Aβ, can play a significant role in AD pathogenesis.

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Keywords: Alzheimer’s disease; AICD; Seizure; Amyloid precursor protein; Axonal sprouting; Neuronal network

Alzheimer’s disease (AD) is a progressive neurodegenerative disease characterized by the histopathological hallmarks of neurofibrillary tangles and senile plaques made up of amyloid-β (Aβ) peptides (Price and Sisodia, 1998). The neurological symptoms of AD include loss of short-term memory, confusion, disorientation to time and place, and loss of initiative, which finally lead to complete loss of cognition (Hardy and Selkoe, 2002; Zheng and Koo, 2006). A significant number of AD patients also show alterations in electroencephalograms (EEG) and the presence of silent seizures (Amatniek et al., 2006; Cabrejo et al., 2006; Snider et al., 2005). Although the exact cause of AD is not known, a large number of studies show that Aβ peptides play a pivotal role in disease pathology. However, it has become increasingly clear that AD etiology is highly complex and that other factors also contribute to disease pathogenesis (Pimplikar, 2009; Small and Duff, 2008).

Aβ peptides are produced by proteolytic processing of amyloid precursor protein (APP). The presenilin-mediated cleavage of APP that causes extracellular release of Aβ also results in intracellular generation of APP intracellular Domain (AICD). We and others have shown that AICD regulates gene expression, alters cell signaling pathways, and causes deleterious effects in tissue culture cells (Gilberto et al., 2008; Muller et al., 2008). We also showed that AICD-overexpressing transgenic mice recapitulate AD-like features such as hyperphosphorylation of tau, impaired memory, and neurodegeneration (Ghosal et al., 2009). Interestingly, AICD transgenic mice also exhibit nonconvulsive seizures and aberrant EEGs (Vogt et al., 2009), which are also seen in human AD and mouse models of AD (Del Vecchio et al., 2004; Minkeviciene et al., 2009; Palop et al., 2007). Work by Mucke and colleagues (Palop et al., 2007) highlighted the occurrence of seizures as a significant pathological feature of AD and characterized the role of Aβ peptides in causing aberrant EEGs and the histopathological changes in...
hippocampal circuits associated with neural hyperactivity. Here we show that elevated levels of AICD in transgenic mice also cause seizure-associated histopathological changes that are similar, but not identical to, those seen in other mouse models of AD. Aging or exposure to excitotoxic stress in young transgenic mice exacerbates these pathological features, suggesting that elevated levels of AICD render neurons hypersensitive to environmental insults and aging.

1. Methods

1.1. Animals

Transgenic mice coexpressing the AICD and Fe-65 (FeCy25) or Fe-65 alone (Fe27) under the control of the CAMKIIa promoter were previously described (Ghosal et al., 2009; Ryan and Pimplikar, 2005). Wild-type littermates were used as controls. For all experiments mice were used at 3–5 or >18 months of age. All experiments were approved by the Institutional Animal Use and Care Committee of The Cleveland Clinic.

1.2. Kainic acid injections

Kainic acid (Sigma) was injected intraperitoneally (i.p.) at a dose of, 20 mg kg$^{-1}$ (Vogt et al., 2009). Mice given sublethal doses were sacrificed after 3 days and transcendally perfused with ice cold PBS followed by 4% paraformaldehyde and processed as described below.

1.3. Immunohistochemistry

Hemi brains were fixed in PBS containing 4% paraformaldehyde overnight, sunk in 30% sucrose and embedded in OCT. Sagittal sections (30 μm) were cut and every 12th section was used for immunohistochemistry. Sections were washed in PBS, treated with 3% H$_2$O$_2$ in PBS, and incubated in blocking solution (5% normal goat serum in PBS containing 0.01% Triton-X) for 1 hour at room temperature. Sections were incubated with primary antibody against neuropeptide Y (NPY) (1 : 1,000) or calbindin-D28k (1 : 500) overnight at 4 °C followed by respective secondary antibodies (1 : 250) and developed using the ABC kit (Vector Laboratories). Microscopy was performed using a Leica DMR microscope equipped with a CCD camera for bright field imaging.

1.4. Quantitative analysis of immunoreactivity

Digital images of brain sections were obtained from 5 to 7 sagittal sections per mouse with a Magnafire-Firewire digital camera (Optronics, Goleta, CA) on a Leica DMR microscope (Leica, Germany). Integrated optical density (IOD) was measured using ImageJ software (National Institutes of Health (NIH)) from a defined area of 0.51 × 0.44 mm (0.223 mm$^2$) in the mossy fiber terminal region, the molecular layer of the dentate gyrus and the stratum radiatum of the CA1 region. No changes were detected in the stratum radiatum. The IOD of the stratum radiatum was subtracted from the IOD of the mossy fiber region to get a normalized IOD value for the mossy fiber region relative to the background intensity. The mean IOD for each animal was calculated from the normalized IOD for each section (5–7 sections per animal) and used for statistical tests. Image analysis was done blind to the treatment group.

1.5. Timm staining and scoring

Timm staining was performed as described previously for other mouse models of AD (Palop et al., 2007) (Minkeviciene et al., 2009). Briefly, mice were administered an i.p. injection of sodium selenite (Sigma) in 0.9% normal saline at a dose of, 20 mg kg$^{-1}$. After 30 min, mice were deeply anesthetized with Avertin and transcendally perfused with ice cold PBS. Brains were harvested and post-fixed in 4% paraformaldehyde in PBS. Every 12th sagittal section was used for Timm staining. Briefly, sections were washed in PBS and incubated in dark conditions for 90 min in Timm developing solution (30% acacia, 0.11% silver lactate, 0.85% hydroquinone, 2.35% sodium citrate, and 2.55% citric acid). The developing reaction was terminated by repeated washes with water. Sections were counterstained with cresyl violet and mounted with glycerol. Mossy fiber sprouting was scored according to conventional techniques (Cavazos et al., 1991; Nissinen et al., 2001) that were previously reported for other AD mouse models (Minkeviciene et al., 2009; Palop et al., 2007), where the following scale was used: (0) no granules; (1) sparse granules in the supragranular region and the inner molecular layer; (2) evenly distributed granules in the supragranular region and the inner molecular layer; (3) almost continuous layer of Timm-positive staining in the supragranular region and the inner molecular layer; (4) continuous band of Timm-positive staining in the supragranular region and the inner molecular layer; and (5) dense and thick band of Timm staining covering the inner molecular layer. The experimenter was blind to the treatment groups during scoring.

1.6. Statistical analysis

Statistics were performed using GraphPad InStat software (version 4). One way ANOVA was employed and significance was set at $p < 0.05$.

2. Results

We previously showed that AICD transgenic mice exhibit abnormal EEG patterns and seizure-like hyperactivity (Vogt et al., 2009). Seizures can induce reorganization of mossy fibers in the dentate gyrus (Buckmaster and Dudek, 1997) which can lead to further overexcitation resulting in prolonged seizure-like symptoms (Santhakumar et al., 2005). Because FeCy25 mice show aberrant EEG activity, we examined the integrity of hippocampal neural circuits by Timm staining, which stains mossy fiber terminals. 4-Months-old
FeC/H9253 25 mice showed significantly increased Timm-positive sprouting in the inner and outer molecular layers that was absent in age-matched wild-type mice (Fig. 1A,D). At 4 months (A) robust Timm staining was identified in the inner molecular layer (iml) beyond the granule cell layer (gcl) in AICD transgenic mice compared with wild-type mice. Aging (B) further increased sprouting in AICD transgenic mice only with a band of axon collaterals in the iml (arrowheads). KA treatment at 4 months (C) had a similar effect to aging, where robustly increased sprouting (arrowheads) was identified in FeCγ25 mice only. (D–F) Mean mossy fiber sprouting score was assessed from 5 to 6 sections from each animal on a scale from 0–5 (described in detail in Methods section) for wild-type (n = 4), Fe27 (n = 4) and FeCγ25 transgenic mice (n = 4) at 4 month (D), >18 month (E), and 4 month with kainic acid (F). Mossy fiber scoring reveals that in all three conditions FeCγ25 transgenic mice had significantly increased sprouting compared with wild-type and fe27 transgenic mice. Scale bar = 50 μm. *p < 0.05 One-way ANOVA, n = 4 for all groups.

FeCγ25 mice showed significantly increased Timm-positive sprouting in the inner and outer molecular layers that was absent in age-matched wild-type mice (Fig. 1A,D). The sprouting was exacerbated in older (>18 months) FeCγ25 mice compared with age-matched wild-type mice (Fig. 1B, E), such that the mossy fiber collaterals formed a band in the inner molecular layer (Fig. 1B, arrowheads). When young mice were injected with sub-convulsive dose of kainic acid (KA), we noticed intense axonal sprouting in young FeCγ25 mice but not in wild-type mice of the same age (Fig. 1C, arrowheads; F). The sprouting intensity in KA-administered young FeCγ25 mice was comparable to that observed in untreated aged FeCγ25 mice (compare Fig. 1C,1B and 1E to 1 F). By contrast Fe27 mice, which express Fe-65 alone, showed no mossy fiber sprouting and resembled wild-type animals (Fig. 1D–F). These results suggest that elevated levels of AICD induce aberrant sprouting of mossy fiber collaterals that is exacerbated by excitotoxic insults or aging.

Seizures enhance expression of neuropeptide Y (NPY) in mossy fibers, which acts as an antiepileptogenic factor (Lin et al., 2006). Because FeCγ25 mice are prone to seizures we examined the NPY levels in mossy fibers. NPY levels were barely detectable in young mice (4 months) and there were no significant differences between the NPY levels in wild-type or FeCγ25 mice (Fig. 2A, D). By contrast, the NPY levels were significantly higher in aged (>18 months) FeCγ25 mice compared with age-matched controls or young FeCγ25 mice (Fig. 2B, E). We next studied the effects of excitotoxic stress and found that administration of KA caused only a...
modest increase in NPY levels in wild-type and Fe27 mice, while FeCγ25 mice responded with significant upregulation (Fig. 2C, F). Thus, NPY levels are increased in mossy fiber collaterals in aged FeCγ25 mice and resemble those in KA-administered young transgenic mice.

A number of other mouse models of AD also exhibit silent seizures, which have been characterized in greater detail by Mucke and colleagues in hAPP mouse model (Palop et al., 2007). Unlike FeCγ25 mice, hAPP mice over-express APP with familial mutation (Swedish and Indiana)
and show elevated levels of Aβ and exacerbated plaque pathology. These mice also show increased levels of NPY in the mossy fiber terminals as well as in the molecular layer of the dentate gyrus. We next measured NPY immunoreactivity in the molecular layer in AICD transgenic mice and found no detectable presence of NPY in the molecular layer in wild-type, Fe27 or FeCγ25 transgenic mice at 4 month (A), > 18 month (B) and 4 month with kainic acid (C). There was no significant difference in relative NPY IR at 4 months in noninduced animals (A). Aging (B) and KA treatment (C) did not induce NPY expression in FeCγ25 mice. (D–F) Quantification of NPY IR at 4 month (D), > 18 month (E) and 4 month with kainic acid (F). n = 3 for all groups. Scale bar = 100 μm for all.

Fig. 3. Ectopic NPY expression is absent in the dentate gyrus of FeCγ25 mice. (A–C) Relative NPY immunoreactivity (IR) was scored in the molecular layer (m) of dentate gyrus above the granule cell layer (g) in wild-type, Fe27 and FeCγ25 transgenic mice at 4 month (A), > 18 month (B) and 4 month with kainic acid (C). There was no significant difference in relative NPY IR at 4 months in noninduced animals (A). Aging (B) and KA treatment (C) did not induce NPY expression in FeCγ25 mice. (D–F) Quantification of NPY IR at 4 month (D), > 18 month (E) and 4 month with kainic acid (F). n = 3 for all groups. Scale bar = 100 μm for all.

region, there was no increase in NPY levels in the molecular layer of aged FeCγ25 mice and there were no significant differences in the NPY levels in the molecular layer of FeCγ25 mice, wild-type or Fe27 mice (Fig. 3B, E). Similarly, KA-induced excitotoxicity did not increase NPY immunoreactivity in the molecular layer of FeCγ25 mice compared with age-matched wild-type or Fe27 mice (Fig. 3C, F). These results suggest that NPY levels are increased in
mossy fiber collaterals but not in the molecular layer in aged FeC\textsubscript{25} mice or young AICD mice exposed to excitotoxic stress. Thus, unlike hAPP mice, AICD transgenic mice do not show compensatory inhibitory projections that terminate in the molecular layer of the dentate gyrus.

Increased hyperexcitability observed in different APP transgenic mouse models of AD is associated with a loss of calbindin D28k immunoreactivity (Minkeviciene et al., 2009; Palop et al., 2007). More recently, loss of calbindin immunoreactivity has been reported in human epilepsy (Magloczky, 2010). Therefore, we stained brain sections with anti-calbindin antibodies and found reduced calbindin immunoreactivity in the molecular layer of the dentate gyrus in FeC\textsubscript{25} mice compared with wild-type mice (compare Fig 4A–C). At a higher magnification, the reduced number of calbindin-positive neurons in the dentate granule cell layer was visible (Fig 4B v. 4 D). The loss of calbindin immunoreactivity seemed restricted to the hippocampus, since there were no significant changes in calbindin immunoreactivity in the cerebellum of AICD transgenic mice (Fig 4E, F). We also found that administration of KA reduced calbindin immunoreactivity in the hippocampus of both wild-type mice and FeC\textsubscript{25} mice (Fig. 4G, I). At higher magnification, the loss of calbindin-positive cells in wild-type mice was apparent (Fig. 4H), whereas it was more severely reduced in AICD mice (Fig. 4I) compared with age-matched wild-type controls (Fig. 4H). By comparison, the loss of calbindin in cerebellum in KA-treated mice was less severe (Fig. 4K, L). Together with our previous findings, these observations show that seizure activity in AICD mice is accompanied by a loss of calbindin-D28k immunoreactivity.

3. Discussion

A large body of literature shows that A\beta plays a pivotal role in AD pathogenesis. Nonetheless, an increasing number of studies indicate that factors other than A\beta also contribute to AD pathogenesis (Holmes et al., 2008; Simon et al., 2008). Our earlier findings that AICD transgenic mice exhibit several AD-like pathological features including aberrant electrical activity, silent seizures, and sensitivity to excitotoxic stress (Vogt et al., 2009) suggest that AICD could be one such important factor. In the present study, we extended these observations and demonstrated that AICD mice show mossy fiber sprouting beginning at 3–4 months of age and increased expression of NPY in aged animals. These pathologies were exacerbated after excitotoxic insults, which resulted in the neural circuits from young AICD mice administered with KA resembling those from old untreated AICD mice. Aberrant axonal sprouting (Cotman et al., 1990; Masliah et al., 1993) and elevated levels of NPY have been observed in AD patients and mouse models of AD (Diez et al., 2003; Minkeviciene et al., 2009; Palop et al., 2007; Ramos et al., 2006) and have been attributed to increased A\beta levels (Palop et al., 2007). Because the pathological features observed in AICD mice occur without increased A\beta levels or alterations in APP processing (Ghosal et al., 2009), our studies suggest that both AICD and A\beta can contribute to axonal sprouting and can increase NPY levels in AD brains.

Although AICD transgenic mice and other mouse models of AD (such as hAPP or APdE9) show sprouting and reorganization of hippocampal neural circuits, there are subtle but significant differences in the observed pathologies. The axonal sprouting in AICD transgenic animals resembles that in temporal lobe epilepsy; in both cases, mossy fiber collaterals terminate as a band in the inner molecular layer and cause further excitation of adjacent granule cells. By contrast, sprouting of mossy fibers in hAPP transgenic mice (which overexpress Swedish K670N/M671N and Indiana V717F mutations in APP) terminates on basket cells and results in proper maintenance of excitatory-inhibitory balance (Cavazos et al., 1991; Palop et al., 2007). By contrast, APdE9 mice, expressing both APP\textsubscript{695}K595N/M596L and PS1-M671L and Indiana V717F mutations in APP, show convulsive seizures in aged AICD transgenic mice (Vogt et al., 2009) but not in hAPP mice. This difference could also explain why AICD transgenic mice (Ghosal et al., 2009) but not the hAPP or APdE9 (Minkeviciene et al., 2009; Palop et al., 2007) mice show hippocampal neuronal loss during aging. Neuronal loss is associated with an epileptic phenotype (Buckmaster and Dudek, 1997; Cavazos and Sutula, 1990) and the cumulative effect of long-term overexcitation may contribute to neurodegeneration in AICD transgenic mice. However, similar to hAPP and APdE9 models, we observed a loss of calbindin immunoreactivity in the hippocampus of AICD transgenic mice.

Our findings that aged AICD mice show elevated levels of NPY agree with reports in AD patients and mouse models of AD (Diez et al., 2003; Minkeviciene et al., 2009; Palop et al., 2007; Ramos et al., 2006). NPY is known to be an anticonvulsant and antiepileptic (Baraban et al., 1997) and its levels in mossy fibers are elevated because of a feedback response to hyperactivity (Nadler et al., 2007). However, unlike hAPP-J20 mice, the increase in NPY expression in AICD mice was not observed in the molecular layer of the dentate gyrus where NPY counteracts overexcitability and protects mice from generalized seizures (Palop et al., 2007). Instead, the NPY expression pattern in AICD mice resembled that of normal temporal lobe epilepsy (Vezzani et al., 1999) and other mouse models of AD with seizure-like phenotypes (Diez et al., 2003; Minkeviciene et
al., 2009). It should be noted that the expression of transgene in AICD mice is driven by a CaMKII/H9251 promoter, which becomes active primarily in the excitatory neurons of forebrain and hippocampus. Thus, its effects could be more pronounced on excitatory neurons, leading to sustained overexcitation and tonic-clinic seizures at older ages (Vogt et al., 2009). This could also account for the differences in seizure semiology and morphological differences between AICD transgenic mice and other APP transgenic mouse models that are based on increased Aβ levels. We have

![Fig. 4. FeCy25 mice display a loss of calbindin immunoreactivity. (A–D) FeCy25 transgenic mice (A) at 4 month of age displayed reduced calbindin-D28k immunoreactivity in the molecular layer (m) of dentate gyrus compared with age-matched wild-type mice (C). (B, D) Higher magnification images of the dentate molecular layer show a loss of calbindin-positive cell bodies in the dentate granule cell (g) layer of in FeCy25 transgenic mice (B) compared with wild-type mice (D). (E–F) No loss of calbindin immunoreactivity in the cerebellum (crb) was detected for either wild-type (E) or FeCy25 transgenic mice (F). (G–J) KA treatment reduced calbindin immunoreactivity in both wild-type (G, H) and FeCy25 transgenic mice (I, J) at 4 month specifically in the dentate gyrus. (K–L) Almost no loss of calbindin immunoreactivity in the cerebellum (crb) was detected for either wild-type (K) or FeCy25 transgenic mice (L). Scale bar = 100 μm (A, C, G, I), 25 μm (B, D, H, J) and 50 μm (E, F, K, L).]
analyzed EEG patterns in the R1.40 mouse model of AD (expressing APPsweK670N/M671L fAD mutation) that shows similar levels of AICD as the FeC52/H9253 mice but displays higher levels of Aβ. We found that R1.40 mice also showed a seizure phenotype, similar to the AICD transgenic model (Ryan and Pimplikar, 2005; Vogt et al., 2009).

Another important observation of the present study is that the elevated levels of AICD render neurons hypersensitive to stress associated with aging or excitotoxicity in vivo. AICD was shown to increase sensitivity in neurons in vitro in cell culture (Giliberto et al., 2008) and our study extends these findings in vivo. We previously showed that a low dose of KA produced moderate convulsive seizures in wild-type mice but caused lethality in AICD transgenic mice (Vogt et al., 2009). Consistent with these findings, young AICD mice subjected to excitotoxic insult developed exacerbated histopathological features that resembled those seen during aging. Similarly, young AICD mice showed normal levels of NPY and no neurodegeneration, while aged AICD mice or young AICD mice injected with KA displayed higher NPY levels (this study) and a severe loss of CA3 neurons (Ghosal et al., 2009). Thus, it could be argued that the effects of acute excitotoxic stress are equivalent to those seen during aging. These findings are consistent with the observations that aging is the most significant risk factor for ad.

In summary, we report that AICD transgenic mice show histopathological changes in the hippocampus similar to those seen in AD patients and mouse models of AD. This study further confirms that elevated levels of AICD render neurons vulnerable to stress associated with aging or excitotoxicity. Uncovering the precise mechanisms underlying the effects of AICD will be important since it may offer new targets for therapeutic interventions.

Disclosure statement

The authors declare no actual or potential conflicts of interest.

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