Sodium Channel Cleavage Is Associated with Aberrant Neuronal Activity and Cognitive Deficits in a Mouse Model of Alzheimer's Disease

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BACE1 is the rate-limiting enzyme that cleaves amyloid precursor protein (APP) to produce the amyloid β peptides that accumulate in Alzheimer's disease (AD). BACE1, which is elevated in AD patients and APP transgenic mice, also cleaves the β 2-subunit of voltage-gated sodium channels (Nav β 2). Although increased BACE1 levels are associated with Nav β 2 cleavage in AD patients, whether Nav β 2 cleavage occurs in APP mice had not yet been examined. Such a finding would be of interest because of its potential impact on neuronal activity: previous studies demonstrated that BACE1-overexpressing mice exhibit excessive cleavage of Nav β 2 and reduced sodium current density, but the phenotype associated with loss of function mutations in either Nav β -subunits or pore-forming α -subunits is epilepsy. Because mounting evidence suggests that epileptiform activity may play an important role in the development of AD-related cognitive deficits, we examined whether enhanced cleavage of Nav β 2 occurs in APP transgenic mice, and whether it is associated with aberrant neuronal activity and cognitive deficits. We found increased levels of BACE1 expression and Nav β 2 cleavage fragments in cortical lysates from APP transgenic mice, as well as associated alterations in Nav1.1 α expression and localization. Both pyramidal neurons and inhibitory interneurons exhibited evidence of increased Nav β 2 cleavage. Moreover, the magnitude of alterations in sodium channel subunits was associated with aberrant EEG activity and impairments in the Morris water maze. Together, these results suggest that altered processing of voltage-gated sodium channels may contribute to aberrant neuronal activity and cognitive deficits in AD.

Introduction

Alzheimer's disease (AD) is characterized by progressive decline in memory and cognition (Blennow et al., 2006). Although the amyloid precursor protein (APP) and amyloid β (A β) peptides cleaved from it play a major role in AD pathophysiology (Bertram et al., 2010; Mucke and Selkoe, 2012), the precise mechanisms leading to cognitive deficits are poorly understood.

AD is associated with a 5- to 10-fold increase in seizure incidence (Amatniek et al., 2006; Lozsadi and Lerner, 2006), and transgenic mouse models of AD exhibit brain-wide aberrant neuronal and epileptiform activity (Hsiao et al., 1995; LaFerla et al., 1995; Moechars et al., 1999; Lalonde et al., 2005; Palop et al., 2007; Minkeviciene et al., 2009; Vogt et al., 2009; Harris et al.,

2010; Roberson et al., 2011; Sanchez et al., 2012). Aberrant activity induces hippocampal dysfunction and memory deficits; modulating aberrant activity and seizure susceptibility modulate cognitive deficits in APP mice (Palop et al., 2007; Roberson et al., 2007, 2011; Sanchez et al., 2012; Verret et al., 2012) and patients with amnesic mild cognitive impairment, a condition that confers risk for AD (Bakker et al., 2012). Although seizures were previously thought to be secondary to disease progression, aberrant activity and/or seizures may directly contribute to cognitive deficits early in disease progression.

An observation of relevance is that BACE1, the rate-limiting enzyme in A β production, also cleaves the β 2-subunit of voltage-gated sodium channels (Nav β 2) in cortical regions (Kim et al., 2007). Similar to APP, Nav β 2 is a single transmembrane domain protein that is cleaved by BACE1 to produce a C-terminal fragment (CTF) subsequently cleaved by γ -secretase to release an intracellular domain (ICD) (Wong et al., 2005; Kim et al., 2007). Nav β 2-ICD translocates to the nucleus and triggers expression of the pore-forming 1.1 α subunit (Nav1.1 α). BACE1 overexpression in transgenic mice or cells results in excessive cleavage of Nav β 2 and surplus expression of Nav1.1 α that is intracellularly retained, leading to reduced surface levels (Kim et al., 2007). Such alterations decrease action potential propagation and neuronal activity (Chen et al., 2002; Lopez-Santiago et al., 2006; Kim et al., 2007). The *in vivo* phenotype associated with loss-of-function

Received May 14, 2012; revised March 3, 2013; accepted March 13, 2013.

Author contributions: B.F.C., J.T.B., A.D.R., M.N.P., P.H.R., and J.C. designed research; B.F.C., S.C.L., H.-P.L., R.N., N.B., X.Z., A.H., J.T.B., A.W., and J.C. performed research; J.T.B. contributed unpublished reagents/analytic tools; B.F.C., S.C.L., N.B., X.Z., J.T.B., A.D.R., and J.C. analyzed data; M.N.P., P.H.R., and J.C. wrote the paper.

We thank A. Silverio for technical assistance and Dr. E. Masliah for human brain tissue from the Alzheimer's Disease Research Center at the University of California San Diego. The JC lab is grateful for the support of the Alzheimer's Association and the Margaret Q. Landenberger Research Foundation.

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DOI:10.1523/JNEUROSCI.2325-12.2013

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Figure 1. Voltage-gated sodium channel alterations in APP mice and AD patients. **A**, Cleavage of Navβ2 by BACE1 and γ-secretase and alterations in Nav1.1α. Modified from Kim et al. (2007). **B**, Increased BACE1 expression and Navβ2-CTFs in cortical lysates from 5- to 6-month-old APP compared with NTG mice (middle and bottom). Navβ2-CTFs are further cleaved by γ-secretase to produce an ICD that translocates to the nucleus to increase Nav1.1α expression, which is also increased in APP mice (top). **C**, Incubation of the anti-Navβ2 antibody with blocking peptide eliminated the bands around 50 and 13 kDa, indicating that these bands are Navβ2-specific bands. **D–F**, Quantification of blots in **B**, $n = 8–10$ /genotype. **G**, Reduced surface levels of Nav1.1α in APP mice. Cortical slices were biotinylated and immunoprecipitated for cell-surface proteins. APP mice had less surface Nav1.1α despite increased total levels, quantified in **H**, $n = 6$ /genotype. **I**, Relationship between surface levels of Nav1.1α and performance in the hidden platform portion of the water maze, a hippocampal-dependent memory task. **J**, Cortical lysates from AD patients also exhibit increased Navβ2-CTFs and total Nav1.1α levels, similar to previously published (Kim et al., 2007). * $p < 0.05$, ** $p < 0.01$.

mutations in Navβ-subunits or α-subunits is epilepsy (Catterall, 2010). Disinhibition from loss of GABAergic activity could contribute, but loss of neuronal activity in other populations may also cause imbalances between excitation and inhibition.

BACE1 is elevated in AD patients and APP mice (Fukumoto et al., 2002; Holsinger et al., 2002; Sun et al., 2002; Yang et al., 2003; Li et al., 2004; Tesco et al., 2007; Zhao et al., 2007; Hebert et al., 2008), and increased BACE1 levels/activity are associated with Navβ2 cleavage in AD patients (Kim et al., 2007). However, whether Navβ2 cleavage occurs in APP mice has not been examined. Such a finding would be of interest because of implications for neuronal activity. Moreover, because APP mice have no experimental manipulations of BACE1 levels/activity, such a finding would demonstrate that increased levels of Aβ can drive increases in BACE1 levels that are sufficient to exert physiological consequences.

Materials and Methods

Transgenic mice. Tg2576 mice express human APP_{Swe} (Hsiao et al., 1996). PSAPP mice express human APP_{Swe} and human presenilin_{M146L} (Holcomb et al., 1998). Mice were singly housed with *ad libitum* access to food and water. For brain harvesting, mice were flush-perfused transcardially with PBS. Hemibrains were frozen on dry ice for biochemistry or postfixed in 4% paraformaldehyde for immunohistochemistry. All procedures were conducted in accordance with the Wyeth Research Institutional Animal Care and Use Committee and the Thomas Jefferson University Institutional Animal Care and Use Committee.

Western blot analysis. The cortex from one hemibrain of each mouse was isolated and homogenized with a Polytron tissue homogenizer in ice-cold RIPA buffer containing (in mM): 50 Tris-HCl, pH 7.6, 150 NaCl, 2 EDTA, 0.1% SDS, 0.01% NP-40, Protease Inhibitor Cocktail (Roche). Lysates were centrifuged at $5000 \times g$ for 10 min. Equal amounts of protein were resolved by SDS-PAGE on 4–12% gels and transferred to nitrocellulose, and probed with antibodies against Navβ2 (Alomone), Nav1.1α (Alomone), BACE1 (Cell Signaling Technology), or actin (Millipore). For testing of anti-Navβ2 specificity, antibody was incubated with control peptide antigen (Alomone) at ratio of 1:5 (antibody/peptide) or PBS for 1 h at room temperature, before incubation with the blot. IR-dye-conjugated secondary antibodies were used for detection and quantification using a LI-COR Odyssey infrared imaging system.

Immunohistochemistry. Brain sections (30 μm) were stained with avidin-biotin/immunoperoxidase using rabbit-anti-calbindin (Swant), rabbit-anti-NPY (ImmunoStar), and rabbit-anti-Navβ2 (Alomone), and signal detected using biotinylated goat-anti-rabbit antibody (Vector Laboratories). Diaminobenzidine was used as a chromagen. For double-labeling experiments, brain sections were stained with rabbit-anti-Navβ2 and mouse anti-GAD67 (Millipore), and signal was detected using FITC-conjugated goat-anti-rabbit and rhodamine-conjugated goat-anti-mouse secondary antibodies (Jackson ImmunoResearch). Immunofluorescence was visualized with confocal microscopy (Olympus Fluoview 1000). Nontransgenic (NTG) and APP

mice were processed simultaneously, and stained sections were analyzed as described previously (Chin et al., 2005) using AxioVision and NIH ImageJ, or MetaMorph, to acquire and quantify images.

Cell surface biotinylation assay. Mice were cervically dislocated and brains rapidly placed into ice-cold Krebs' solution containing (in mM): 125 NaCl, 4 KCl, 1.25 KH₂PO₄, 10 glucose, 1.5 MgSO₄, 26 NaHCO₃, 1.5 CaCl₂. A Vibratome was used to cut 350 μm brain slices. Slices were equilibrated in Krebs' solution bubbled with 95% oxygen and 5% carbon dioxide in a 30°C water bath before biotinylation in 1 mg/ml biotin solution (Pierce) at 4°C. Cortical/hippocampal regions were microdissected and homogenized as above, and equal amounts of protein were incubated with NeutrAvidin-agarose beads (Pierce) to pull down biotinylated proteins.

Morris water maze. Male Tg2576 and NTG mice (5 months old, 9–10 per genotype) were tested in a white circular pool (1.5 m diameter) filled with 22°C water made opaque with nontoxic white paint. The platform

(15 cm diameter) used for hidden platform training sessions was submerged 1 cm below the water surface. The platform (30 cm diameter) used during cued platform sessions was at water surface level and had a flowerpot in the center. During cued platform training, a curtain encircled the pool preventing visual access to extra-maze cues. Performance was video-tracked and analyzed using EthoVision (Noldus Information Technology).

Hidden platform training involved 8 trials/d over 7 d. Sixty-second trials were separated by a 10 min intertrial interval. During acquisition trials, platform location remained constant and mice entered the pool from randomized locations. Latency and distance traveled to the platform were analyzed. After the last training trial, the platform was removed and mice were given a 60 s probe trial test. Mice were then tested in cued platform sessions. Latency to reach the platform was measured for four trials, each starting from different locations, to assess visual acuity and motor performance. The experimenter was blinded to genotypes during testing.

EEG surgery and recordings. Mice (5–7 months) were implanted with an indwelling electrode (Plastics One) consisting of three polyimide-coated wires with bare tips, implanted at a depth of 1 mm from the dorsal surface of the brain over the hippocampus (2.5 mm posterior, 2.0 mm lateral from bregma) and in frontal cortices (1 and 2 mm rostral from hippocampal wire). This implant provided a differential signal recorded at 1 kHz, amplified 1000 $\times$, and bandpass filtered from 0.1 to 500 Hz with a 60 Hz notch filter in line, using a CED Micro1401 data acquisition system and Spike2 (Cambridge Electronic Design) software. EEG signals were interpolated to 128 Hz and bandpass filtered from 1 to 64 Hz. Power spectral densities (PSDs) were calculated with fast Fourier transform to convert the waveform data into a power spectrum with 0.5 Hz resolution. For each frequency bin (0.5 Hz), the value of power in that frequency was divided by the sum of all power in all frequencies up to 64 Hz. To calculate the amount of time spent at >6 Hz (time in high frequency), PSDs were generated every 30 s for each recording. The signal processing ensured integer values representing the dominant frequency (DF) in hertz for each 30 s epoch. Average DF was calculated for each mouse from each DF in each 30 s epoch (3600/30 s = 120 epochs) in its recording. The time in high frequency was calculated by summing the number of 30 s epochs that had a DF $\geq$ 6 Hz and dividing it by the total number of 30 s epochs (total time of the recording) for each mouse.

Results

Alterations in BACE1 expression, cortical Nav β 2 cleavage, and Nav1.1 α expression in APP mice

Previous studies demonstrated that BACE1 overexpression results in increased cleavage of Nav β 2, and altered expression and surface localization of Nav1.1 α as illustrated in Figure 1A (Kim et al., 2007). Because BACE1 levels are increased in AD patients and other lines of APP mice (Fukumoto et al., 2002; Holsinger et al., 2002; Sun et al., 2002; Yang et al., 2003; Li et al., 2004; Tesco et al., 2007; Zhao et al., 2007; Hebert et al., 2008), we examined whether APP mice exhibit similar alterations in voltage-gated sodium

Figure 2. Increased numbers of Nav β 2-positive nuclei in cortex of APP mice. Nav β 2-positive nuclei were assessed in brain sections from APP mice after immunohistochemical staining with an antibody that recognizes Nav β 2-ICD. **A**, APP mice exhibit increased numbers of Nav β 2-positive nuclei in cortical regions. Arrow points to a Nav β 2-positive nucleus. Inset, Hematoxylin counterstain demonstrates nuclear Nav β 2 localization (arrow). Arrowhead, Nucleus with no Nav β 2. **B**, Quantification of nuclear Nav β 2 staining in APP and NTG mice, $n = 7$ –9/genotype. **C**, Quantification of Nav β 2 staining in brain sections from PSAPP mice reveals a significant increase in Nav β 2-positive nuclei also in this mouse model, $n = 5$ –7/genotype. **D**, **E**, Double-labeling of Nav β 2 and GAD67 in brain sections from APP mice. Confocal images of Nav β 2 and GAD67 immunostaining demonstrate that some Nav β 2-positive nuclei are found in cells that also express GAD67 (**D**, arrowheads), but others do not express GAD67 (**E**, arrows). * $p < 0.05$, *** $p < 0.001$.

channels. Using Western blot analysis of cortical lysates, we confirmed that the 5- to 6-month-old, pre-plaque APP mice used in this study also exhibit increased BACE1 expression (Fig. 1B,D). We also found increased levels of Nav β 2 CTFs produced by BACE1 cleavage, as well as increased expression of Nav1.1 α (Fig. 1B,E,F). Incubation of the anti-Nav β 2 antibody with control peptide antigen before exposure to the membrane demonstrated that the bands $\sim$ 45–50 kDa and 13 kDa are specific Nav β 2 bands (Fig. 1C) similar to those observed in previous studies (Messner and Catterall, 1985; Kim et al., 2007; Gersbacher et al., 2010). Surface biotinylation assays of hippocampal slices demonstrated that the increased expression of Nav1.1 α was retained intracellularly, and was not detected on neuronal cell surfaces (Fig. 1G,H). Thus, the aberrantly expressed Nav1.1 α subunits likely remain nonfunctional, as previously described (Kim et al., 2007). These alterations in sodium channel levels appear to have functional consequences, because we found an inverse relationship between surface (functional) expression of Nav1.1 α and performance in

Figure 3. Aberrant neuronal activity in APP mice. **A**, EEG traces demonstrate increased amplitude and frequency of brain activity in two representative NTG and two APP mice at 5–7 months of age. **B**, Radar plots show time (2 h) as a continuous parameter, and frequency from 0 to 10 Hz in concentric circles. **C–E**, APP mice exhibit longer durations of higher frequency activity than NTG mice (**C**), which is associated with increased total Nav1.1 α levels (**D**) and increased Nav β 2 cleavage (**E**) ($n = 7$ –9/genotype). **F, G**, APP mice exhibit decreased calbindin expression in dentate gyrus (**F**) and ectopic NPY expression in mossy fibers (**G**). **H, I**, Calbindin expression in the dentate gyrus correlated inversely (**H**) and NPY expression in mossy fibers correlated positively (**I**) with the EEG parameter of time spent in high-frequency. * $p < 0.05$.

the hidden platform portion of the Morris water maze, a hippocampal-dependent memory task (Fig. 1I). We did not find sodium channel alterations in hippocampal lysates from APP mice (data not shown), suggesting that enhanced cortical Nav β 2 cleavage influences hippocampal function. Total levels of Nav1.1 α and Nav β 2 CTFs also appeared to be increased in cortical lysates from brains of AD patients relative to controls (Fig. 1J), similar to that previously demonstrated (Kim et al., 2007).

Nuclear accumulation of Nav β 2 cleavage fragments in GABAergic and non-GABAergic neurons

To identify neuronal populations susceptible to Nav β 2 cleavage, we looked for brain regions with enhanced nuclear localization of Nav β 2-ICD fragments produced after cleavage by BACE1 and

γ -secretase. The antibody used in this study recognizes a 19 aa sequence at the most C-terminal portion of Nav β 2, a region contained in the ICD fragment that translocates to the nucleus (Wong et al., 2005; Kim et al., 2007). We found an increase in Nav β 2-positive nuclei in the cortex of APP mice (Fig. 2A,B). The increase was not specific to a certain region of cortex, although it was qualitatively more obvious in somatosensory and entorhinal cortical areas. This result suggests that cortical neurons are subject to increased Nav β 2 cleavage. We also found increased numbers of Nav β 2-positive nuclei in the cortex of transgenic mice that express both mutant human APP and mutant human presenilin (PSAPP mice), another well characterized transgenic mouse model of AD, demonstrating that these findings are not unique to only one transgenic model of AD (Fig. 2C). To evaluate whether the neurons that exhibited increased nuclear levels of Nav β 2 are GABAergic and/or glutamatergic, we performed double-labeling for Nav β 2 and GAD67 (Fig. 2D,E), a marker of GABAergic interneurons that is expressed by nearly 100% of interneurons in the neocortex (Rudy et al., 2011). Confocal imaging indicated that both GABAergic and non-GABAergic neurons exhibited nuclear accumulation of Nav β 2.

Aberrant neuronal activity is associated with sodium channel alterations in APP mice

Epileptiform activity and/or seizures have been previously reported in some lines of APP mice with high levels of A β ; however, the data for Tg2576 mice are variable (Hsiao et al., 1995; LaFerla et al., 1995; Moechars et al., 1999; Lalonde et al., 2005; Palop et al., 2007; Oberheim et al., 2008; Minkeviciene et al., 2009; Roberson et al., 2011; Sanchez et al., 2012; Verret et al., 2012). Therefore, to determine whether the sodium channel alterations and memory deficits we observed might be related to aberrant neuronal activity, we performed video-EEG recordings to verify that these mice exhibit aberrant neuronal activity. We found that 5- to 7-month-old APP mice exhibited spike-wave discharges and abnormal EEG patterns (Fig. 3). APP mice exhibited longer durations of higher frequency brain activity, suggesting increased synchrony (Fig. 3A–C), although they did not exhibit obvious spikes or seizures. We confirmed that APP mice with epileptiform activity exhibited increased Nav β 2 cleavage and increased total levels of Nav1.1 α (Fig. 3D,E), although there was no direct one-to-one correlation between time spent in high-frequency and Nav β 2 CTFs ($p = 0.27$, $n = 7$) or Nav1.1 α ($p = 0.78$, $n = 7$) for individual APP mice (data not shown). The lack of one-to-one correlation between Nav1.1 α /Nav β 2 and time spent in high-frequency may reflect the fact that lysate from the

entire cortex was used to measure Nav1.1 α /Nav β 2, rather than specific regions that might contribute more directly to epileptiform activity. APP mice exhibited decreased calbindin expression in the dentate gyrus and ectopic NPY expression in mossy fibers of the hippocampus, both of which are well documented hallmarks of chronic excitotoxicity that serve neuroprotective roles but that impair neuronal function (Molinari et al., 1996; Vezzani et al., 1999; Nägerl et al., 2000; Palop et al., 2007) (Fig. 3*F,G*). Calbindin expression correlated inversely, and NPY expression correlated positively, with the EEG measure of time spent in high-frequency (Fig. 3*H,I*). Decreased calbindin expression in the dentate gyrus is also observed in AD patients, and the magnitude of the decrease corresponds with the degree of dementia (Palop et al., 2003). In APP mice, decreases in calbindin in the dentate gyrus also correlate tightly with hippocampal-dependent learning/memory deficits (Palop et al., 2003).

Together, these results suggest that aberrant sodium channel cleavage may contribute to aberrant neuronal activity and cognitive deficits in APP mice. BACE1 inhibitors may provide therapeutic benefit by both reducing A β production, and normalizing sodium channel function and aberrant neuronal activity.

Discussion

A role for network dysfunction in AD-related cognitive deficits was recently highlighted in a number of studies, underscoring the need to identify cellular mechanisms that contribute to altered neuronal activity (Palop et al., 2007; Minkeviciene et al., 2009; Roberson et al., 2011; Bakker et al., 2012; Sanchez et al., 2012; Verret et al., 2012). Here we report increased Nav β 2 processing and reduction of Nav1.1 α surface expression in cortex of APP mice. Intracellular retention of Nav1.1 α may result from impairment of trafficking due to intracellular accumulation of Nav1.1 α subunits; from elevated BACE1 activity in APP mice, which impairs trafficking; or from a relative loss of Nav β 2, which is necessary for surface expression of Nav1.1 α (Kovacs et al., 2010). These alterations decrease sodium channel density and action potential propagation in other systems (Chen et al., 2002; Lopez-Santiago et al., 2006; Kim et al., 2007), suggesting that activity in cortical neurons may be impaired in APP mice. Because Nav β 2 is expressed in inhibitory and excitatory neurons (Lai and Jan, 2006), the network consequence of increased Nav β 2 cleavage could be very different depending on the cell type in which it occurs. Our studies suggest that both glutamatergic and GABAergic neurons are susceptible to enhanced Nav β 2 processing. Moreover, although normally Nav1.1 α is more highly, but not exclusively, expressed in inhibitory interneurons (Gong et al., 1999; Ogiwara et al., 2007), its expression could also be altered in excitatory neurons after increased processing of Nav β 2.

A recent study in another line of APP mice demonstrated decreased levels of Nav1.1 α only in parvalbumin-positive interneurons, which led to impaired interneuron function and aberrant neuronal activity that could be normalized by overexpressing Nav1.1 α in interneurons (Verret et al., 2012). The mechanisms by which Nav1.1 α alterations arise in that model remain to be defined. Although our studies demonstrate increased rather than decreased total levels of Nav1.1 α , we found that surface Nav1.1 α levels are reduced (Fig. 1*G,H*). Thus, both studies demonstrate decreased levels of functional Nav1.1 α in cortex of APP mice. These findings indicate that sodium channels can be regulated by more than one mechanism, and present an important target by which neuronal activity might be modulated in APP mice.

Coordinated cortical activity and oscillations strongly influence function of the hippocampus, which receives information

about spatial contexts from a number of cortical areas including entorhinal, perirhinal, retrosplenial, posterior parietal, and prefrontal cortex (Buzsáki, 2002; Gordon, 2011; Vann and Albasser, 2011). Encoding of spatial information depends on the integration of information from these dynamically interconnected regions, and is modulated by attention (Muzzio et al., 2009; Derdikman and Moser, 2010). Indeed, we found that alterations in cortical Nav1.1 α levels were associated with aberrant EEG activity, and correlated inversely with performance in the water maze (Figs. 1*I*, 3).

The APP mice used here have no experimental manipulations of BACE1 levels/activity. Thus, overexpression of APP/A β is sufficient to initiate a pathway leading to sodium channel cleavage, possibly through regulation of BACE1 levels (Bourne et al., 2007; Shimmyo et al., 2008). Mice that overexpress BACE1 exhibit not only sodium channel alterations but also impairments in learning and memory (Rockenstein et al., 2005; Kim et al., 2007). However, seizures and epileptiform activity have not yet been described in BACE1-transgenic mice. Ablation of BACE1 leads to opposite changes in sodium channel levels as well as epileptiform activity and cognitive deficits, indicating that a balance of BACE1 activity must be maintained to preserve normal neuronal activity and network function (Ohno et al., 2004; Kobayashi et al., 2008; Hitt et al., 2010; Hu et al., 2010; Kim et al., 2011).

Recent studies have suggested several cellular mechanisms for aberrant neuronal activity in AD and related mouse models, including depolarized resting membrane potential and increased excitability of cortical pyramidal neurons (Minkeviciene et al., 2009), impaired inhibition (Busche et al., 2008), and decreased activity of parvalbumin-positive interneurons due to loss of voltage-gated sodium channel subunits (Verret et al., 2012). Together, these and other mechanistic studies are beginning to identify the range of cellular mechanisms by which neuronal activity and network function may become dysregulated in AD. Further studies are necessary to understand how and when these cellular alterations occur during disease progression and how they fit together in the manifestation of aberrant neuronal activity in AD and related models.

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