Pathogenesis of sporadic Alzheimer's disease by deficiency of NMDA receptor subunit GluN3A

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Abstract

The Ca^{2+} hypothesis for Alzheimer's disease (AD) conceives Ca^{2+} dyshomeostasis as a common mechanism of AD; the cause of Ca^{2+} dysregulation, however, is obscure. Meanwhile, hyperactivities of NMDA receptors (NMDARs), the primary mediator of Ca^{2+} influx, are reported in AD. GluN3A (NR3A) is a NMDAR inhibitory subunit. We hypothesize that GluN3A is critical for Ca^{2+} homeostasis, its deficiency is pathogenic for AD. Cellular, molecular and functional changes were examined in GluN3A knockout (KO) mice during aging. The GluN3A KO mouse brain displayed age-dependent moderate but persistent neuronal hyperactivity, elevated intracellular Ca^{2+}, neuroinflammation, impaired synaptic integrity/plasticity, and neuronal loss. GluN3A KO mice developed olfactory dysfunction followed by psychological/cognitive deficits prior Aβ/tau pathology. Memantine at preclinical stage prevented/attenuated AD syndromes. AD patients' brains show reduced GluN3A expression. We propose that chronic "degenerative excitotoxicity" leads to sporadic AD, while GluN3A represents a primary pathogenic factor, an early biomarker and an amyloid-independent therapeutic target.

Graphical Abstract

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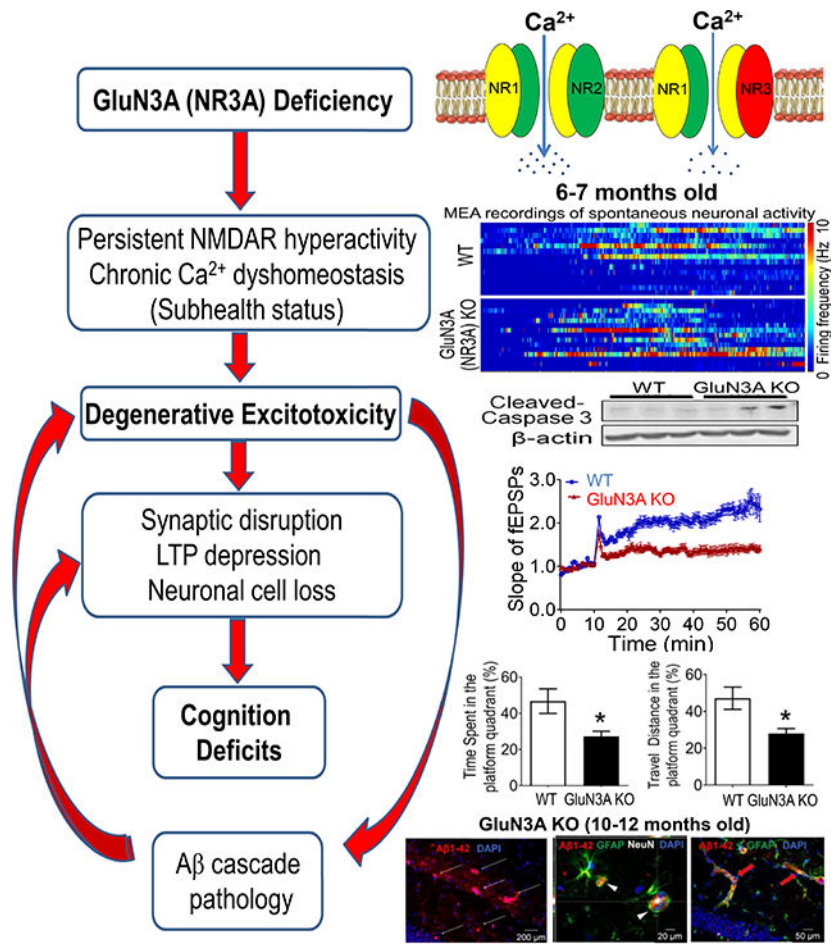
PART 1: NARRATIVE

1.1. Objective and research goal

Alzheimer's disease (AD) affects 1 in 10 people of age 65 and older, while the exact pathogenic factor remains ambiguous. Among all proposed hypotheses for AD, the "Ca^{2+} hypothesis" has gained increasing supports with the observations that Ca^{2+} dysregulation is a common and prevalent pathophysiology in AD. However, the initiating mechanism that causes lifelong alterations in Ca^{2+} homeostasis leading to neuronal dysfunction and cognition decline has not been identified. Here, we aim to disclose a root mechanism that can explain the origin of Ca^{2+} dyshomeostasis and its link to "degenerative excitotoxicity" responsible for age-dependent cognition decline and amyloid cascade pathology.

1.2. Background and rationale

Alzheimer's disease (AD) is characterized by the development of age-dependent dementia accompanied by the formation of amyloid β (Aβ) plaques and neuronal fibrillary tangles (NFTs) [1–4]. Synaptic disruption and neuronal loss in the neocortex and hippocampus as well as brain atrophy are structural characteristics of AD [3]. In addition to cholinesterase inhibitors and the NMDA receptor (NMDAR) antagonist memantine (MEM) approved by FDA as symptomatic treatments for moderate to severe patients, there is no treatment that can cure or prevent AD. Extensive research has identified three typical genetic mutations including amyloid precursor protein (APP) [5], presenilin 1 (PS1) and PS2 [6], which are responsible for genetically inherited forms of AD (familial AD or FAD). FAD shows early onset and represents less than 1–2% of total AD cases [7]. Most AD patients (>95%) are sporadic type of AD (SAD) featuring late onset [8] with no clearly identified etiology. The genetic mutations in FAD support the "amyloid cascade hypothesis", which suggests that AD pathogenesis is initiated by overproduction of Aβ and/or failure of its clearance, upstream of tau dysregulation and dementia [3, 9]. While the Aβ and tau pathology remains the current diagnostic standard of AD, they are poorly correlated with symptoms of AD such as progressive memory loss and cognitive decline; i.e. individuals may have significant Aβ plaques and tau tangles in the hippocampus and neocortex but show no sign of cognitive deficits [10–13]. Nevertheless, the majority of AD investigations have been performed on transgenic rodent models expressing FAD genes. The failure of clinical translation of experimental therapies based on the amyloid hypothesis and the lack of correlation between brain amyloid levels and cognitive status have led to the proposition to reevaluate or even reject the conventional, although very popular, amyloid cascade hypothesis so as to explore alternative pathogenic mechanisms [13–16]. Different hypotheses such as the "inflammatory hypothesis" and the "oligomer/soluble Aβ hypothesis" modified from original amyloid hypothesis have been proposed alongside others for a number of years [17]. However, so far few of these hypotheses have been able to account for all the hallmark pathologies and symptoms of AD or result in clinical therapies to show significant therapeutic effects against the disease progression.

Meanwhile, the "Ca^{2+} hypothesis" for AD/dementia has gained increasing supports from basic and clinical research [18–21]. The Ca^{2+} hypothesis was initially proposed by Khachaturian, predicted that a sustained dysregulation of cellular Ca^{2+} homeostasis could disrupt neuronal function and lead to neurodegenerative diseases such as AD [22]. This hypothesis has been endorsed by consistent observations that neuronal Ca^{2+} dysregulation is a common mechanism in the development of AD in both animal models and clinical studies [18, 19, 23–27]. Perturbations or increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) were also demonstrated in cells and organoids from AD patients and in clinical imaging studies [28–30]. It is thus widely recognized that Ca^{2+} dysregulation is an important pathophysiology of familial and sporadic AD, although the causal mechanism that triggers the $[Ca^{2+}]_i$ disturbance has not been clearly defined.

Ca^{2+} is a prevalent second messenger regulating numerous cellular functions, including, but not limited to, neuronal excitability, synapse integrity and plasticity associated with learning and memory [31]. The Ca^{2+} level in the extracellular space is typically maintained between

1 and 2 mM, whereas the resting cytosolic concentration of free Ca^{2+} in neurons is tightly regulated around the 100 nM range [32]. This substantial Ca^{2+} gradient is critical for normal neuronal activity and cell viability [33, 34]. Upon excitation, Ca^{2+} enters neurons via Ca^{2+} permeable channels and receptors on the plasma membrane and is quickly sequestered by intracellular buffering systems including Ca^{2+} binding proteins and internal organelles, such as the endoplasmic reticulum (ER), mitochondria, and lysosomes [35, 36]. Many studies have demonstrated their interactions with amyloid cascades, thereafter, resulting in Ca^{2+} dysregulation.

In the validation of the Ca^{2+} hypothesis of AD, we have focused on the regulation of Ca^{2+} influx via the NMDA receptor that plays primary roles in neuronal activity, synaptic transmission/plasticity, cognitive function as well as excitotoxicity of glutamatergic neurons [37, 38]. Under pathological conditions, overactivation of NMDARs and increased $[Ca^{2+}]_i$ are biological signals that trigger a chain of molecular and cellular events leading to acute and delayed cell death and pathological consequences such as synapse loss, neurovascular damage, mitochondrial dysfunction, and failure of neural network communications [38–40]. Compelling evidence has shown hyperactivity of NMDARs in AD [28–30], but the precise mechanism that causes persistent NMDAR hyperactivity and whether this NMDAR misbehavior has a causal relationship to AD development remain unidentified.

1.3. Historical evolution and updated hypothesis

Excitotoxicity was initially described in neuronal cultures and animal models of stroke 30 years ago in investigations of glutamate-induced toxicity to neurons [34, 41, 42]. It is defined as excessive exposure to the neurotransmitter glutamate or overstimulation of its membrane receptors, leading to massive Ca^{2+} influx and intracellular Ca^{2+} overload [43, 44], resulting in acute neuronal injury i.e. necrotic cell death within several hours to a few days after the insult [34, 40, 45]. The Ca^{2+} hypothesis for AD resembled the similar concept of Ca^{2+} dysregulation, predicting that excitotoxicity is responsible for neurodegenration developed in the AD brain [18, 21, 24]. However, distinctions between the acute and chronic forms of excitotoxicity have not be explicitly defined.

We propose the concept of "degenerative excitotoxicity" whereby some ulterior cause of Ca^{2+} disregulation is the root of a slight but sustained increase in $[Ca^{2+}]_i$. The resultant "calciumopathy" yields brain region specific impairment that is not immediately injurous but induces enduring perturbations of multiple Ca^{2+}-dependent cascades and generates a chronic pro-AD state prone to programmed cell degeneration involving synaptic disruption, recurring inflammation, metabolic interruption, aberrant autophagy, apoptosis, and other cytotoxic consequences including the activation of amyloid cascades [46–49]. According to this modification to the Ca^{2+} hypothesis, there must be a pathogenic mechanism that exists during presymtomatic stage and persists to advance to age-dependent abnormal consequenses. It is also hypothesized that for a prolonged period of time (months in rodents and years in humans), the AD susceptible brain can still be functional under a "suboptimal health or subhealth" status [50]. This status could exist prior to the development of any brain pathology yet exert significant stress to neuronal infrastructure, brain matablism, and neural network functions. Aβ cascades have been proposed as triggers for excitotoxity in

AD [51–53]. The proposition, however, cannot account for how neurodegeneration could develop before Aβ related pathologies in many AD cases. Our investigation is an initial effort to reveal the primary pathogenic mechanism mediated by continual hyperactivities of NMDAR in the deficiency of its unique inhibitory subunit GluN3A.

Ionotropic glutamate receptors, includind NMDA, AMPA, and kainate receptors, serve as important mediators of excitatory synaptic transmission in the brain [37, 54]. Of these, NMDAR plays a major role in Ca^{2+} influx of cellular physiology and pathophysiology [38, 39]. NMDARs comprise the GluN1 (NR1) subunit, GluN2 (NR2) subunits (GluN2A-2D), and a pair of GluN3 (NR3) subunits (GluN3A and GluN3B) [55]. Functional NMDARs are heterotetramers composed of two glycine/D-serine-binding GluN1 subunits paired with two glutamate-binding GluN2 subunits, or often with one GluN2 and one inhibitory GluN3 subunit [56]. GluN3A and 3B act in a dominant-negative manner to restrain receptor activity. Heterogeneous expression of NMDARs with a GluN3 subunit induces smaller NMDA currents with reduced Ca^{2+} permeability [57–60]. The GluN3A expression in dendritic spines of glutamatergic neurons implies that GluN3A can influence synaptic stability, receptor assembly, and functional activity of these neurons [61]. Different from GluN3A, GluN3B mainly expresses in motor neurons. GluN3A knockout (KO) cortical neurons exhibit increased NMDA currents and dendritic spine density, supporting that GluN3A plays a significant role in synaptic modulation/plasticity [57]. There is a high level of sequence homology (93%) between human and rodent GluN3A/3B, implying that their function is likely similar between mammalian species [62]. The lack of gene equivalents in *D. melanogaster* and *C. elegans* suggests that GluN3 emerged during evolution of vertebrates in the need for a more complicated and more precisely controlled nervous system. We propose that GluN3 genes are essential and critical regulators for optimal NMDAR function and sustained Ca^{2+} homeostasis in the mammal's brain.

The control of NMDAR activity is imparitive for normal brain function. Repeated NMDAR activation enhances postsynaptic Ca^{2+} entry, which is a pre-requisite for AMPA receptor (AMPAR) activation and subsequent synaptic long-term potentiation (LTP) [38]. This essential role of NMDAR makes it uniquely positioned to mediate synaptic plasticity, forming a molecular and cellular basis for learning and memory [38]. Abbarrent NMDAR activities can otherwise cause synapse loss and synaptic disruption [63]. The toxicity is principally mediated by excessive Ca^{2+} entry via over-activated NMDARs [41, 64]. Meanwhile, how the NMDAR activity is persistantly altered in the aging brain and its regulatory mechanism in AD progression are poorly understood. Current research in this area has exclusively examined GluN1 and GluN2; little attention has been paid to a possible role of GluN3 in AD pathophysiology.

In rodents, the GluN3A expression is high in the developing brain but lower in adults [65, 66]. Our previous data indicate that despite this drop off, GluN3A remains prevalent in the adult mouse brain and exerts significant neuroprotective effects against excitotoxicity after ischemic stroke among other important functional roles [45, 67, 68]. In humans the distribution of GluN3A is significant and widespread in both fetal and adult brains [69, 70]. It was identified that there are "native NR1-NR2A/B-NR3A assemblies in adult human CNS", and GluN1 (NR1) and GluN3 (NR3) "behave similarly with regard to receptor

assembly and trafficking" [70]. Thus, GluN3A can play vital regulatory roles in both young and adult human physiology and pathophysiology.

Human studies have identified that GluN3 encoding genes *GRIN3A* and *GRIN3B* are highly heterogeneous; around 2–10% people worldwide carry mutated *GRIN3* variants including null alleles with complete deficiency (G*RIN3A* mutates in ~2% Han-Chinese and Japanese, no information available for *GRIN3A* in other countries; *GRIN3B* variants in 10% of populations in European-Americans and some other countries) [71–73]. A clinical study of AD patients investigated genetic risk factors based on *GRIN3A* and *GRIN3B* alleles [74]. Two single nucleotide polymorphisms, 3104 G/A (rs10989563) and 3723 G/A (rs3739722) in *GRIN3A* and 2 *GRIN3B* polymorphisms were probed and the genetic variation of the *GRIN3A* was suggested to be a risk factor for AD [74]. It is worth mentioning that the percentage of people with *GRIN3* mutations is in close proximity to the prevalence of AD (1.6 – 6.4%) among general populations in different countries [75]. Nevertheless, there is no further information about a possible correlation between mutation/dysfunction of *GRIN3A/GRIN3B* and AD.

The FDA approved use of MEM as a treatment for moderate-to-severe AD provides a support for the clinical significance of NMDAR regulation in AD [76–78]. The essential role played by NMDARs in early Ca^{2+} dysregulation suggest that MEM could delay or halt the progression of early AD. Indeed, several clinical trials in mild AD/dementia patients support this prediction although this approach is still under debate [79–85]. In this investigation, we examined a primary pathogenic role of persistent NMDAR hyperactivity mediated by the deficiency of its inhibitory subunit GluN3A in AD development, leading to cognition decline well before the formation of Aβ plagues and tau pathology. Data from the GluN3A KO mouse shed lights on the mystery of what is the initiating mechanism or the mysterious switch for a chronic pro-AD Ca^{2+} dysregulation and whether it is possible to delay, ameliorate or prevent AD progression at an early phase of the disease.

PART II: CONSOLIDATED REULTS AND STUDY DESIGN

2.1. Consolidated results and study design

In brain slices from GluN3A KO mice, we performed MultiElectrode Array (MEA) recordings and tow-photon Ca^{2+} imaging. Comparing to young adult GluN3A KO brains, the aging KO brain exhibited hyperactivities of spontaneous firing accompanied with moderate but sustained elevations of resting $[Ca^{2+}]_i$. Meanwhile, the aging KO brain showed reduced presynaptic protein synapsin and impaired synaptic plasticity in the hippocampus compared to wild type (WT) age-matched controls. Significant increases of Ca^{2+}-dependent signaling proteins such as CamKII and PKCα were identified in the hippocampus and/or cortex. Inflammatory activities such as increased TNF-α, reactive astrocytes and microglia cells persisted in the aging brain of GluN3A KO mice but not in normal aging controls. In age-dependent manners, GluN3A deficiency culminated in neuronal loss due to programmed cell death and recapitulated the disease symptoms including early onset of olfactory dysfunction followed months later by neuropsychological and cognitive deficits, which were not seen in WT controls. AD pathological hallmarks, i.e. Aβ production/deposition and tau hyperphosphorylation developed in the cortex and hippocampus in a much-delayed

fashion several months after the onset of cognition decline. Consistent with the hypothesis that NMDAR hyperactivity is a pathogenic factor, MEM treatment at a clinically relevant dose (10 mg/kg/day in drinking water for 3 months) starting at the preclinical stage of 3-months old prevented or attenuated psychological and cognitive symptoms in GluN3A KO mice compared to vehicle controls. Of clinical significance, Western blot analysis of AD patient's brain samples revealed lower levels of GluN3A in the cortex compared to normal aging subjects. We propose that a chronic state of hyperexcitability-induced "degenerative excitotoxicity" due to the GluN3A deficiency is sufficient to initiate AD evolution in an age-dependent manner. The long-term GluN3 modulation of NMDARs begins early in life, which implies early biomarkers, a wide therapeutic window for intervention, and potential targets for sporadic AD. The GluN3A KO mouse provides a novel model for studying the Ca^{2+} hypothesis in SAD development. The information of degenerative excitotoxicity obtained from this SAD mouse model may help to better understand other neurodegenerative diseases involving disrupted Ca^{2+} homeostasis.

PART III: DETAILED METHODS AND RESULTS

3.1. Methods and animals

GluN3A KO mice and wild type (WT) counterparts were tested. There was no change in the expression of other NMDAR subunits [74]. Detailed information of these mice was described previously [57, 62]. Genotyping of animals was performed as needed [67, 86]. Detailed experimental methods are provided in Supplemental Materials.

3.2. Neuronal hyperactivity and impairments of synaptic plasticity in the GluN3A KO brain

In brain slices from adult GluN3A KO mice of 6–12-months old (roughly equal to 35–60 years old humans), firing of hippocampal neurons were recorded using the MultiElectrode Array (MEA) system. Spontaneous neuronal activities were monitored for 10 min in 58 hippocampal locations. GluN3A KO hippocampal neurons indeed displayed constant higher frequencies of firings than that in age-matched WT brains (Fig. 1A–1B).

Synaptic disruption is a key pathological step in the early development of AD and proposed to be a Ca^{2+}-dependent cellular degeneration in AD [87]. Western blotting showed that in brains from 3-months old mice, there was no difference in the expression level of the presynaptic protein synapsin between GluN3A KO group and age-matched WT group (Fig. 1C). At 7 months of age, however, a significantly lower level of synapsin was detected in the hippocampus of GluN3A KO mice comparing to WT controls (Fig. 1C). There was no significant difference in the expression of synaptophysin and PSD-95 between WT and KO brains (Suppl. Fig. 1A and 1B).

We previously reported that young adult GluN3A KO mice (2–4-month old) exhibited enhanced LTP compared to age-matched WT mice [68]. To examine whether the chronic hyperexcitability state and the synapsin decrease with aging might disrupt synaptic functions, LTP in the hippocampal slice from aging GluN3A KO mice (6–7-month old) was recorded. In striking contrary to young animals, LTP in the hippocampus of older ages

was evidently suppressed (Fig. 1D). The impaired LTP induction persisted in brain slices of aging GluN3A KO mice of 9–12-month old (Fig. 1E). Paired-pulse facilitation (PPF) was recorded to detect whether the deteriorated synaptic plasticity was attributable to a pre-synaptic abnormality. The PPF, induced by various inter-stimulus intervals, confirmed a defect in synaptic facilitation of transmitter release in the hippocampus of aging GluN3A KO mice (6–12-month old) compared to age-matched WT brains (Fig. 1D and 1E).

3.3. Disrupted Ca^{2+} homeostasis in the GluN3A KO brain

To verify whether the chronic hyperactivity of NMDARs might disrupt tonic Ca^{2+} homeostasis, the basal level of $[Ca^{2+}]_i$ in brain slices was measured using Fura-2-AM two-photon imaging (Fig. 2A–2B). Average $[Ca^{2+}]_i$ values of hippocampal CA1 and dented gyrus (DG) cells of GluN3A KO mice were generally higher, but statistically not significant compared to WT cells due to large variations (Table 1). In the Fura-2 ratio measurement, the value in DG neurons of GluN3A KO mice (10–12-month old) was significantly greater than that of WT neurons (Fig. 2B). We compared the percentage of cells with $[Ca^{2+}]_i$ higher than 200 nM. The percentage of high $[Ca^{2+}]_i$ cells showed a strong indication of increase in CA1 of 6–7-month old GluN3A KO mice (P=0.058 vs. WT). High $[Ca^{2+}]_i$ cells in DG of 10–12-month old GluN3A KO mice was significantly increased compared to age-matched WT mice (Fig. 2C).

3.4. Ca^{2+}-dependent molecular signaling and inflammatory factors in GluN3A KO mice

In the hippocampus of GluN3A KO mice of 6–7-month old, the ratio of phosphorylated Ca^{2+}-calmodulin-dependent kinase II (pCaMKII) against total CaMKII was significantly elevated, while the expression of several Ca^{2+}-dependent signals such as PKCα, calpain, and calbindin all showed trends of increases (Suppl. Fig. 1C and 1D). At older ages of 10–12-month old, the expression of pCaMKII and PKCα remained significantly higher or showed strong trends in the hippocampus and cortex of GluN3A KO mice comparing to WT controls (Fig. 2D–2F). Thus, the GluN3A KO brain presented a unique environment of persistent hyperexcitability, subtle but significant elevation of basal $[Ca^{2+}]_i$, impaired synaptic plasticity of pre-synaptic defect, and specific Ca^{2+}-dependent signaling activities.

Disruption of Ca^{2+} homeostasis is associated with chronic inflammation, inducing productions of inflammatory cytokines and nitric oxide (NO)[88]. The pro-inflammatory factor TNFα significantly increased in the hippocampus of GluN3A KO mice of 6- and 12-month old, although interleukin-1β (IL-1β) and CCL2 (MCP) were unaltered (Fig. 3A–3C). The level of IL-6, an anti-inflammatory factor, was not changed at younger ages, but markedly decreased at 12-month old (Fig. 3D). GluN3A KO mice of 12-month-old displayed enhanced expression of inducible NO synthase (iNOS) (Fig. 3E). The NF-κB signaling, an upstream pathway in neuroinflammation[89], showed an early upregulation with increased NF-κB (p65) in the GluN3 KO hippocampus (Fig. 3F). Meanwhile, more GFAP-positive astrocytes and IBA-1-positive microglia were observed in the hippocampus of GluN3A KO mice of 6–12-month old (Fig. 3G–3H).

3.5. Neuronal loss in the aging GluN3A KO brain

We previously reported neurodegeneration of olfactory sensory neurons in GluN3A KO mice of 2–3-month old [67]. In the present investigation, significant TUNEL-positive cells were detected in the olfactory bulb of 10–12-month old GluN3A KO mice but not in age-matched WT controls (Suppl. Fig. 2A). In adult (6–7-month old) mice of both groups, there was no significant cell death in the hippocampus (Suppl. Fig. 2B–2C, Fig. 3I–3K) and cortex (data not shown). In aging (10–12-month old) GluN3A KO mice, however, NeuN-positive cells significantly decreased in the CA3 region (Fig. 3I–3K). Western blotting identified a moderate but significant increase of apoptotic marker cleaved caspase-3 in the GluN3A KO cortex of 10–12-month old compared to age-matched WT mice (Fig. 3L). The pro-caspase-3 level was unchanged (Suppl. Fig. 2D–2E).

3.6. Olfactory dysfunction and psychological alterations in young adult GluN3A KO mice

Olfactory deficit is an early symptom of neurodegenerative diseases including AD [67]. GluN3A KO mice of different ages were subjected to the olfactory habituation/dishabituation test. When exposed to sequential odors, WT mice increased sniff behavior when different odors were presented, while this increase was absent in GluN3A KO mice of 2–5-month old (Suppl. Fig. 3A–3B). In the buried food test, GluN3A KO mice of 5- and 7-month old took significantly longer latency to find buried food compared to WT mice (Suppl. Fig.3C).

AD patients often develop psychological problems such as anxiety and social barriers [80]. The open field test was used to assess anxious-like behavior [68]. Although WT and GluN3A KO mice of 3-month old behaved normally, aging GluN3A KO mice (7–12-month old) traveled much less in the center area (Suppl. Fig. 3D). In the sociability assay of the three-chamber test, WT mice displayed a preference to visit the chamber hosting a naïve mouse (Suppl. Fig. 3E) and sniffed significantly longer around the stranger (Suppl. Fig. 3F). GluN3A KO mice of 5–12-month old lost these preferences (Suppl. Fig. 3E–3F). In the social novelty test, WT animals spent significantly more time with the novel stranger than with the familiar mouse, whereas GluN3A KO mice showed no preference (Suppl. Fig. 3G–3H).

3.7. Cognition decline in an age-dependent manner in GluN3A KO mice

Progressive memory loss and learning difficulty are hallmarks of AD and dementia. Spatial learning and memory functions were tested using the Morris water maze test [68, 86]. At adult ages of 5–6-month old, GluN3A KO mice started to show slower learning during the 5-day training, although they did not yet have significant difficulties in finding the platform compared to WT mice in the probe trial (Fig. 4A). Significant spatial working memory impairment was seen in aging GluN3A KO mice around and after 7-month old, showing significant less time and travel distance in the platform quarter compared to WT mice (Fig. 4B–4C).

In the novel object recognition test, which requires acquisition, storage and recall of recognition memory of familiar objects [68], WT mice always exhibited preference of novel object over familiar ones, while GluN3A KO mice failed to show such preference

in the retention task 1 hr after training (Fig. 4D). The impaired recognition memory was detected as early as 5-month old and continued with aging. The contextual and cued memory was evaluated by scoring freezing behavior 24 hrs after mice were trained with the conditioned stimulus-unconditioned stimulus (CS–US) pairing. Twenty-four hrs after conditioning, GluN3A KO mice of 5-month old presented significantly less freezing behavior than age-matched WT mice after reintroducing to the conditioning context and tone cue, suggesting poor fear memory (Fig. 4E–4F). GluN3A KO mice did not show deficit in short-term spatial memory in the Y maze spontaneous alternation test (Suppl. Fig. 4).

3.8. Spontaneously developed amyloid and tau pathology in the elderly GluN3A KO brain

There was little Aβ deposition in the 3-month old young adult brains of WT and GluN3A KO mice (Suppl. Fig. 5A). Immunohistochemical assays using the Aβ antibody against 1–42 peptides detected a few Aβ spots in the CA1 region of 6–7-month old GluN3A KO mice, which is not significant compared to age-matched WT controls (Fig. 5A–5B). Significant depositions of endogenous Aβ overlaid with neurons, reactive astrocytes and vasculatures were readily visible in the hippocampus of GluN3A KO mice of 10–15-month old of both sexes but not in age-matched WT mice (Fig. 5A–5C).

In Western blot analysis, the expression of amyloid precursor protein (APP) was similar between WT and GluN3A KO mice at 6–7-month old, while significantly increased APP was detected in the hippocampus of GluN3A KO mice of 10–12-month old (Suppl. Fig. 5B–5C). Meanwhile, no increases of tau phosphorylation were found in the hippocampus and cortex of younger GluN3A KO mice of 6–7-month old, with one exception that the total tau expression noticeably increased in the cortex (Suppl. Fig. 6A–6D). In 10–12-month old GluN3A KO brains, the total tau protein remained to be elevated in the cortex, and hyperphosphorylation was detected in several common phosphorylation sites of tau protein in both the hippocampus and cortex (Fig. 5D–5F). A further analysis on the ratio of *p*-tau against the total tau verified hyperphosphorylation of *p*Tau-Ser199/201 in the hippocampus of GluN3A KO mice (Suppl. Fig. 6E–6F). Thus, Aβ and tau pathology emerged several months after cognition decline (~5 months old) in the GluN3A null animal.

3.9. Age-dependent AD-like symptoms in female GluN3A KO mice

Female GluN3A KO mice aged at 2–5-month old showed poor performance than WT females in the buried food and olfactory habituation/dishabituation test (Suppl. Fig. 7A–7B). In the water maze test, female GluN3A KO mice aged at 7–10-month displayed slower learning in the training phase (Suppl. Fig. 7C). Consistently, female GluN3A KO mice developed impaired recognition/visual memory and contextual fear memory detectable in the novel object and fear conditioning tests (Suppl. Fig. 7D–7E).

3.10. Early memantine treatment prevented olfactory and cognitive deficits of GluN3A KO mice

In adult GluN3A KO mice of 3-month-old when olfactory dysfunction was detected but no cognition deficits, MEM daily treatment was started at a clinically relevant dosage (10 mg/kg/day in drinking water) [90]. Age-matched GluN3A KO mice and WT mice received drug-free water as vehicle and normal controls, respectively. After 3-month treatments, the

olfactory odor discrimination function of GluN3A KO mice in the MEM group recovered to the normal level of WT mice (Fig. 6A). In the Morris water maze test, MEM-treated mice performed better than vehicle controls in the 5-day training (Fig. 6B). The time spent and travel distance of MEM-treated mice in the platform quadrant tended to be marginal longer compared to vehicle controls (Fig. 6C–6D). In the fear conditioning test, MEM-treated KO mice displayed much improved memory of contextual fear compared to vehicle control mice (Fig. 6E). Cued fear memory was not improved by MEM (Fig. 6F). MEM did not show benefit in the novel objective recognition test that involves visual function and recognition memory [91](Fig. 6G), and no improvements in the three-chamber social test (Fig. 6H–6I) or anxious behavior in the open field test (data not shown).

3.11. Reduced GluN3A expression in the brain of Alzheimer's disease patients

We inspected NMDAR subunit levels in human brain tissues of AD patients and normal aging subjects. In Western blot assay of the frontal lobe, the expression of GluN1, GluN2A, GluN2B and GluN3A subunits showed no significant difference between AD and normal brains (Suppl. Fig. 8A–8B). In the occipital cortex, the GluN2A, 2B and GluN3A expressions were significantly lower in the AD brain while the GluN1 level was similar between AD and normal brains (Suppl. Fig. 8A and 8C).

3.12. Discussion, hypotheses and future direction

Previous investigations on the role of NMDARs in AD pathophysiology have exclusively focused on GluN1 and GluN2 subunits for how they might be affected by the amyloid cascade [92, 93]. We show for the first time that deficiency of GluN3A alone progressively evolves a series of AD-associated pathophysiology and functional deficits before detectable Aβ/tau pathologies. Unlike many transgenic AD mouse models, the GluN3A KO mouse develops virtually all major pathophysiology and age-dependent psychological, cognitive symptoms seen in AD patients. AD-related alterations in the human brain begin years before obvious signs of the disease (i.e. preclinical stage)[94]. The spontaneous progression was also evolved in the GluN3A KO mouse, with enhanced neuronal activities, marginally elevated resting $[Ca^{2+}]_i$, selective increases of Ca^{2+}-regulated signaling molecules such as CamKII, PKCα, and calpain. The lasting disruption of Ca^{2+} homeostasis and activation of specific cellular signaling pathways generate a chronic pro-AD environment in the brain. Although the GluN3A deficiency induced $[Ca^{2+}]_i$ increases did not cause instantaneous acute excitotoxicity, the persistent hyperexcitability of glutamatergic neurons ought to imposes a tonic Ca^{2+} burden conferring a subhealth status that slowly leads to degenerative excitotoxicity. Both male and female GluN3A KO mice similarly develop early onset olfactory dysfunction followed by dementia symptoms and thereafter Aβ/tau pathology. Supporting that continual hyperactivity of NMDARs is a risk/causal factor of AD, daily MEM treatment in GluN3A KO mice starting from the preclinical stage for 3 months ameliorated cognitive decline and even restored previously lost olfactory function. The clinical significance of the GluN3A-associated mechanism is supported by the human data showing reduced GluN3A protein level in the postmortem AD brain.

GluN3A KO mice are viable and fertile. In general, they breed normally and do not show significant phenotypic difference from WT littermates up to young adult age. After reaching

adulthood, functional tests in these animals start to detect impairments in locomotor, olfactory and social activities [67, 68, 95]. Interestingly, on the other hand, enhanced learning/memory and greater LTP induction in hippocampal neurons were detected as a unique feature in early adulthood of GluN3A KO mice [68], which is consistent with hyperactivation of NMDARs before advancing to degenerative excitotoxicity. Beyond this age point (around and after 5-month old in this mouse model), a series of psychological and cognitive deficits as well as depressed LTP gradually cultivate with aging in the absence of GluN3A. It will be interesting for an epidemiological study to examine whether similar unique biphasic cognitive function and behavioral features exist during the lifespan of people who bear mutated *GRIN3* genes or those having been diagnosed with AD or dementia.

GluN3A was observed in the pre- and post-synaptic components of glutamatergic neurons and may regulate transmitter release [96]. In GluN3A KO brain slices, we detected a pre-synaptic deficit in synaptic plasticity in PPF recordings. This is highly consistent with the significant decrease of presynaptic proteins synapsin in the aging GluN3A KO brain. Other studies perceived the location of GluN3A to both extrasynaptic and peri-synaptic membrane domains [61]. This complexity implies that both synaptic and extrasynaptic NMDAR activity must be kept in check for normal brain function. It will be intriguing to delineate the relative role of GluN3A across different synaptic locations in AD development.

The molecular regulation and long-term modulation of NMDAR activity in AD are poorly understood. Based on the popular belief that changes in Ca^{2+} signaling are secondary to deleterious actions of Aβ/tau/PS pathogenesis [97], investigations testing the role of Ca^{2+} in AD have been predominantly focused on the relationship between Aβ/tau/PS and Ca^{2+} regulation. Specifically, investigations have closely inspected interactions between the amyloidogenic pathway with membrane channels, receptors and intracellular Ca^{2+} regulatory proteins in the ER and mitochondria [25, 98–100]. Information from these investigations is valuable for understanding FAD and the later stage of SAD, but may not be relevant to the pathogenesis of SAD. Most previous studies on Ca^{2+} dysregulation were performed in transgenic mouse models resembling FAD [25, 98–100]. These observations do not resolve the clinical disconnect between Aβ pathology and cognitive status of aging/aged people. Whether disruption of Ca^{2+} homeostasis is a cause of AD development in an Aβ-independent manner has rarely been questioned or explored. This is likely due to the domination of the amyloid hypothesis and the lack of evidence of an Aβ-independent casual mechanism responsible for chronic Ca^{2+} dyshomeostasis and AD development.

Our results support the Ca^{2+} hypothesis for AD in terms of that NMDAR-mediated Ca^{2+} dyshomeostasis appears to generate a chronic state of degenerative excitotoxicity leading to AD development and progression. In addition, we provide the first clear demonstration that a sustained Ca^{2+} dyshomeostasis and the resulted final common degenerative pathway is due to the deficiency of the NMDAR inhibitory subunit GluN3A. Departing from current view of the Ca^{2+} hypothesis, we show that the disruption of Ca^{2+} regulation is an upstream initiating pathophysiology before and throughout AD progression, but not triggered by the production of any known FAD genes. The amyloid cascade hypothesis has implied that amyloid pathology must precede the clinical symptoms. This is neither the case for

sporadic AD development in many AD patients nor in the GluN3A KO mouse. During the early progression in the SAD brain, there was no evidence of amyloid/tau pathology or alterations of expression levels of APP and ApoE. There were also no evidence supporting crosstalk between the amyloid cascade and Ca^{2+} regulation during this period. Nevertheless, Aβ deposition and tau hyperphosphorylation did occur later in the GluN3A null brain, in harmony with the definition of AD. Presenilin was not measured in these experiments, it may be necessary to delineate whether the production of PS1/PS2 and/or their mutation may contribute to Ca^{2+}-mediated degenerative excitotoxicity.

There are other mechanisms that may affect sustained Ca^{2+} homeostasis. Theoretically, any chronic aberrant cellular and subcellular activities or dysfunction may cause lasting hyperactivity of glutamatergic neurons resulting in sustained Ca^{2+} dysregulation. A few possible examples may include increased expression or function of NMDAR GluN1 and GluN2 subunits, impaired Ca^{2+} buffering systems in cellular organelles including the ER and mitochondria [25, 98–100], soluble Aβ oligomers [101], aberrant neuronal and non-neuronal activities [100], etc. Our observation does not exclude interplays or interactions among signaling pathways such as mitochondrial dysfunction during the AD process. Optimal and constant energy supply is crucial for Ca^{2+} homeostasis and optimal performance of neuronal cells. Chronic burden on energy metabolism may generate a subhealth condition leading to hypoglycemia-like stress followed by neural dysfunction and degeneration. Future investigations should test whether and how these alterations induce the subhealth condition in early and middle AD stages independent of the amyloid cascade and develop possible interventions during different stages of the lifelong process.

There are clear distinctions between degenerative excitotoxicity and acute excitotoxicity in ischemic stroke. These may include 1) temporal differences that an ischemic attack causes sudden eruption of massive Ca^{2+} influx that is far beyond the capacity of intracellular Ca^{2+} buffering pools within a time scale of minutes to hours, leading directly to necrotic death (the dominate form of cell death in stroke) and activation of delayed programmed death cascades in following days if the cells survive from the initial attack [102–104]. In the case of AD, there is no rapid collapse of Ca^{2+} homeostasis while the cellular Ca^{2+} regulatory systems are continuously activated to maintain the resting $[Ca^{2+}]_i$ close to the normal level while gradually losing the capability under the chronic stress; 2) the extent of Ca^{2+} elevation is dramatic after an ischemic insult (from low nM concentrations up to at least μM levels) while degenerative excitotoxicity is associated with much more moderate Ca^{2+} elevation (200 nM range) that is not immediately lethal but chronically stressful and detrimental via downstream signaling; 3) the spatial control of cellular Ca^{2+} in acute excitotoxicity is generally wiped out by Ca^{2+} overflow while during degenerative excitotoxicity intracellular Ca^{2+} pools and buffering systems may retain spatial specificity in subcellular locations, at full strength and then partially, for an extended time period (months in rodents and years in humans). Localized Ca^{2+} signals in dendritic spines and processes are central to proper neuronal activity and synaptic functioning [105, 106]; disruption of localized Ca^{2+} regulation can be a characteristic of degenerative excitotoxicity and requires further investigations with imaging tools; 4) related to the above features, the type of neuronal injury and cell death is significantly different. Necrosis is the dominant form of acute neuronal damage. On the other hand, degenerative excitotoxicity involves delayed and

continuous programmed deteriorations caused by inflammatory factors, metabolic stress, apoptosis, autophagy, and possibly Ca^{2+}-dependent necrotic component. Most likely, the prolonged process allows activation of multiple signaling pathways and injury cascades in the same cells, ultimately evolving to a form of hybrid (mixed) cell death as seen chronically after stroke and energy deficiency [48, 103, 107]. Both neuronal and non-neuronal cells may be affected directly or indirectly by degenerative excitotoxicity throughout AD progression, which needs to be examined in future investigations; 5) in ischemic stroke, Ca^{2+} dysregulation is largely restricted to the ischemic region, while in the AD brain, sustained dysregulation of Ca^{2+} homeostasis may affect multiple brain regions and leads to a global interruption in cellular/structural functions [108]; 6) degenerative excitotoxicity may selectively affect vulnerable brain regions and specific neuronal subtypes due to different GluN3A expression and unique patterns of Ca^{2+} signaling alterations that closely tie to disease symptoms. Thus, regional and cell type specificity may explain pathogenesis for other neurodegenerative diseases such as Parkinson's disease [22, 108]. The possibility that NMDAR-mediated, Ca^{2+} dyshomeostasis-induced degenerative excitotoxicity may have broader implications in neuronal system failure beyond AD and dementia may be considered and explored.

We examined NMDAR subunit expression in human brain samples from AD patients. This initial effort was limited to two cortical regions, yet we still detected a significantly reduced GluN3A protein level in the occipital lobe (OL), where visual information is processed [109]. Visual impairment is a common symptom of AD and prevalently reported as an early sign of the disease [110]. In our study, object recognition deficits were detected in GluN3A KO mice, suggesting impaired vision memory and a potential damage of occipital-temporal connections. The significance of observed reductions of GluN3A and GluN2 levels in the occipital cortex merits further investigations, while possible changes of GluN3A expression in other AD brain regions such as the hippocampus should be examined.

Considering that 2–10% of humans bear mutated/deficient *GRIN3* genes, and similar rates of AD prevalence worldwide [71–73, 75], it appears necessary to study *GRIN3A* variants in different human populations. The relationship between *GRIN3A* mutations/low GluN3A level to AD should be examined and genetic approaches for the GluN3A deficiency may be developed. As a first step, a genetic survey of *GRIN3A* variants and GluN3A expression should be performed in AD patients. Alternative/additional strategies for NMDAR modulations to overcome degenerative excitotoxicity should also be considered. Meanwhile, channelopathy of other Ca^{2+} permeable channels can play roles in the process and remains to be further investigated.

It is recognized that treatments that show even moderate effect in a key domain of AD (cognition, function/behavior) can have significant clinical impacts. Current FDA guideline and clinical trials of MEM therapy are focused on symptomatic treatments for moderate-to-severe AD/dementia, with the Mini–Mental-State-Examination (MMSE) score <20. According to the new evidence in the Ca^{2+} hypothesis, this late stage therapy misses the early pathogenic phase of the disease. More clinical trials with MEM monotherapy in preclinical/mild AD/dementia patients and pre-symptomatic people susceptible to AD should be performed to validate the therapeutic benefits observed in the SAD model. This

can be done with early biomarkers/neuroimaging combined with NMDAR assays and/or olfactory deficits in high risk populations or older people with MMSE score of 21–26. This approach may provide an evidence-based anti-dementia/anti-AD treatment to delay and ameliorate the disease progression. Future basic and clinical research should evaluate how MEM prevents AD/dementia pathology at the cellular and molecular levels. The therapeutic window as well as long-term effects of this treatment should be verified to improve the therapeutic efficacy.

CONCLUSIONS

This investigation provides the first evidence to suggest that a sporadic AD cascade can be initiated by the deficiency of the single NMDAR regulatory subunit GluN3A. The result strongly supports the Ca^{2+} hypothesis that a lifelong moderate but sustained Ca^{2+} overload is a causal pathogenic mechanism of sporadic AD. The GluN3A KO mouse provides a unique animal model closer to sporadic features in most AD patients; and will be useful for mechanistic and therapeutic investigations. We believe the concepts of subhealth status in seemingly healthy individuals and degenerative excitotoxicity in the AD development will help to better explain complicated and sustained conditions in SAD and other neurodegenerative diseases. The therapeutic benefits from preventive MEM treatment are consistent evidence for the pathogenic role of NMDAR hyperactivity and implicate an exciting possibility to delay, ameliorate, or even prevent AD symptoms from early phases of the disease. In addition to pharmacological approaches, genetic strategies may be explored to target the modulation/manipulation of the GluN3 gene *GRIN3A*. Aberrant glutamate release and impaired clearance are potential mechanisms for hyperactivation of NMDARs. More research should focus on glutamate and NMDA induced chronic hyperexcitability and the regulation of sustained Ca^{2+} homeostasis as a unique pathogenic mechanism independent from the production of Aβ or the interaction with amyloid cascades.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Abbreviations:

AD	Alzheimer's disease
APP	Amyloid precursor protein
ApoE	Apolipoprotein E

aCSF	Artificial cerebrospinal fluid
Aβ	β-amyloid
CaMKII	Ca^{2+}-calmodulin-dependent kinase II
CNS	Central nervous system
fEPSP	Field excitatory postsynaptic potentials
HFS	High frequency stimulation
IACUC	Institutional Animal Care and Use Committee
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
$[Ca^{2+}]_I$	Intracellular free calcium concentration
KO	Knockout
LTP	Long-term potentiation
MEM	Memantine
MMSE	Mini–Mental State Examination
MCI	Mild/moderate cognitive impairment
MEA	MultiElectrode Array
NIH	National Institutes of Health
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NMDA receptor	N-methyl-D-aspartate receptor
OL	Occipital lobe
PPF	Paired-pulse facilitation
PACCH	Pro-AD condition of chronic hyperexcitability
TNFα	Tumor necrotic factor α
WT	Wild type

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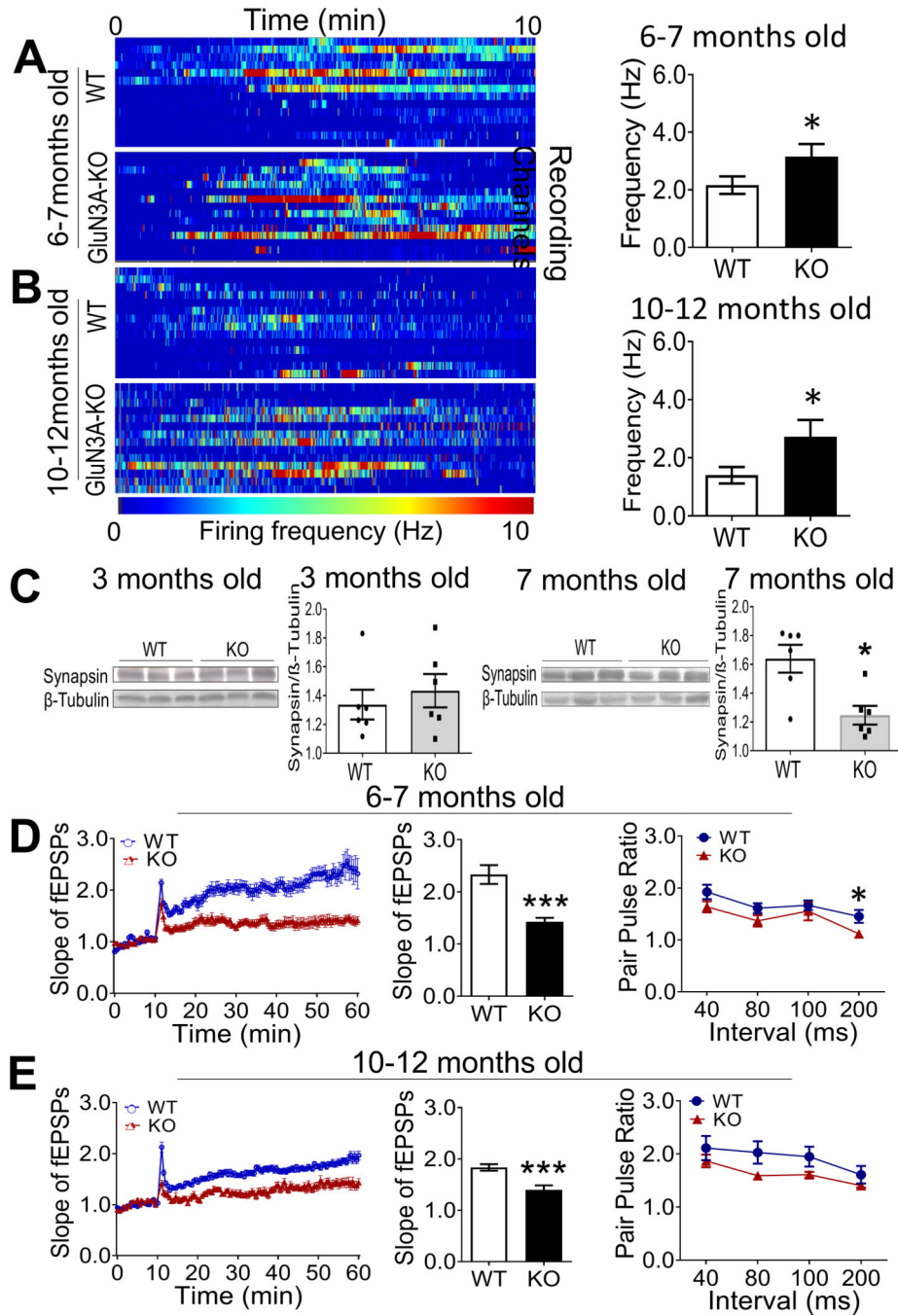
Figure 1. Neuronal hyperactivity and impaired synaptic plasticity in the GluN3A KO brain.
A MultiElectrode Array (MEA) system with 60-electrodes was used to record spontaneous
activities and fEPSP in the brain slices containing hippocampus of WT and GluN3A KO
mice of different ages. **A** and **B**. Heatmaps generated by the frequency of spontaneous
spikes over time in the regular aCSF, are representative recording channels in brain slices
from adult (6–7-month old) and aging (10–12-month old) mice. After stabilization of the
recording for 10 min, spontaneous spikes was monitored for 10 min. Bar graphs show
that the average frequency was significantly higher in GluN3A KO slices of the two age

groups compared to age-matched WT slices (n=4 animals in WT group and 5 in KO group; *P<0.05 vs. WT; Student's *t*-test). **C.** Western blot analysis of the presynaptic protein synapsin in the hippocampus. The expression of synapsin in 3 months old mice was similar between the two groups. Significant reduction of synapsin was seen in GluN3A KO mice at 7-month age compared to WT controls. **D** and **E**. In brain slices from 6–7 (C) and 10–12-month (D) old mice, LTP of fEPSPs in the hippocampus was induced by the typical high frequency stimulation (HFS; 100 Hz, 1 sec) in the CA1 region. The magnitude of LTP in GluN3A KO slices was significantly and constant smaller than that in WT slices. Bar graphs show quantified results at the 50 min time point. On the right, the two-line graphs show paired-pulse facilitation (PPF) induced by stimuli of various inter-stimulus intervals, which is a reflection of presynaptic plasticity. In Two-way ANAVA analysis, the main factor of genetic background (WT vs. KO) showed significant difference (C; *P<0.05), suggested significantly reduced synaptic facilitation/plasticity in GluN3A KO slices at 6–7 months compared to age-matched WT controls. No statistical significance was found between GluN3A KO and WT at 10–12 months old (n=5 animals per group; ***P<0.001, *P<0.05 vs. WT; Two-way ANOVA).
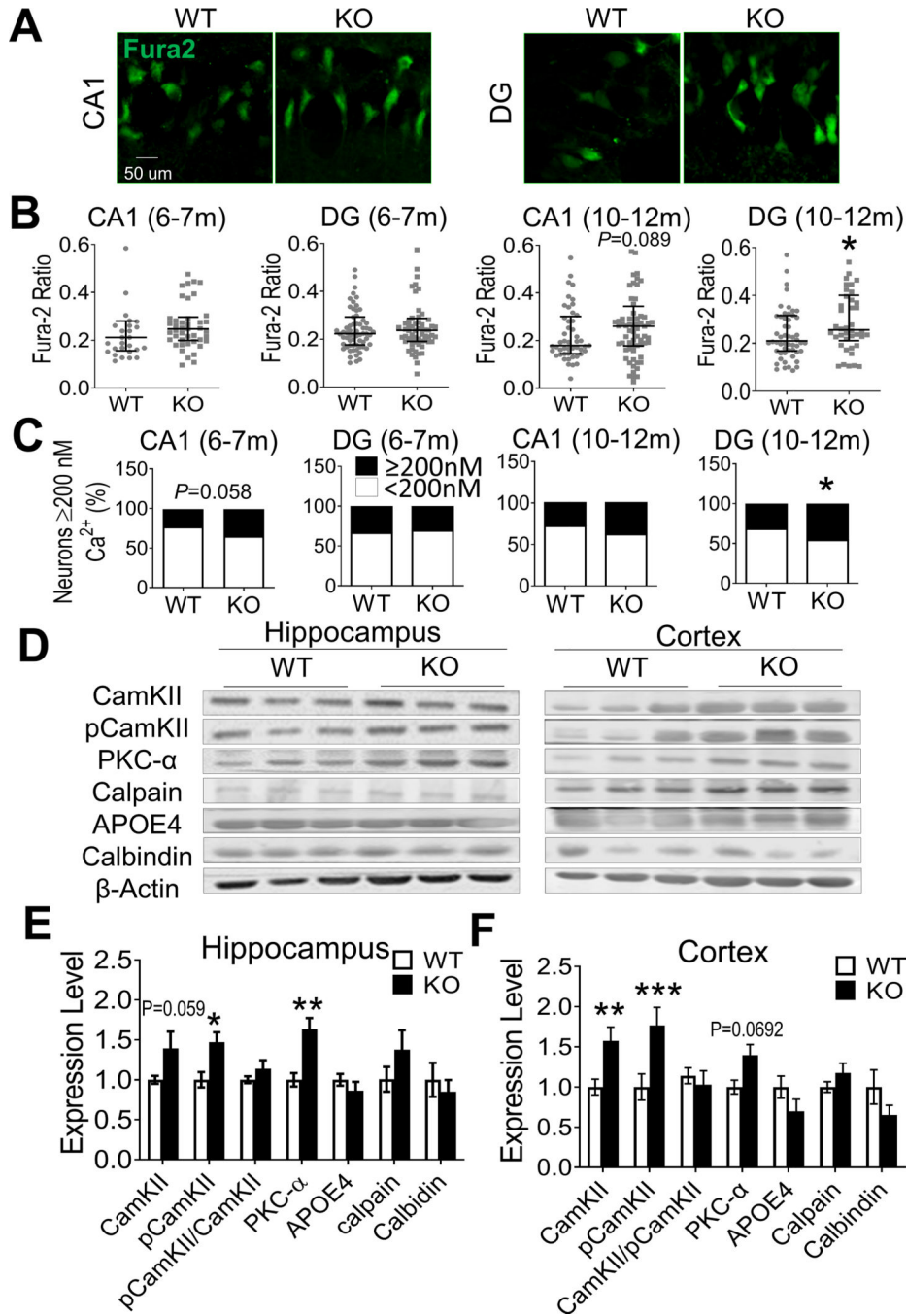
Figure 2. Elevated intracellular Ca²⁺ and Ca²⁺-dependent signaling in the GluN3A KO brain.
Intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ and Ca^{2+}-dependent signaling proteins were measured in the hippocampus and cortex of WT and GluN3A KO mice of 6–12-month old.
A. *In situ* Ca^{2+} imaging using Fura-2AM was performed under a two-photon microscope. Images show Fura-2AM labeled CA1 and DG neurons in brain slices from WT and GluN3A KO mice. **B.** The Fura-2AM ratio measurements of $[Ca^{2+}]_i$ in WT and GluN3A KO neurons at 6–7 month and 10–12-month old n=4 and 5 for WT and KO mice of 6–7-months group, respectively; n=4 animals for both WT and KO mice of 10–12-months group; *P<0.05 vs.

WT; Mann-Whitney U test). **C**. The percentage of CA1 and DG cells with $[Ca^{2+}]_i$ above and below 200 nM in total cells measured. CA1 neurons of 6–7-months old GluN3A KO mice showed a strong trend of greater percentage of high Ca^{2+} neurons compared to WT cells (P=0.05). At the age of 10–12-month old, the percentage of high Ca^{2+} cells was significantly larger than that in WT mice (animal numbers were the same as in B; *P<0.05 vs. WT; Chi-Squared test). **D**. Western blotting of hippocampus and cortex tissues from aging WT and GluN3A KO mice (10–12-month old). **E** and **F**. Summary bar graphs of Western blot data. In the hippocampus, the expression of pCaMKII and PKC-α increased significantly in GluN3A KO mice while CaMKII and calpain showed trends of increase (E). In the cortex of GluN3A KO mice, the expression of CaMKII and pCaMKII was significantly higher while PKC-α remained tentatively higher compared to levels in WT (n=6 animals per group; *P<0.05 vs. WT, **P<0.01 vs. WT; Two-way ANOVA).
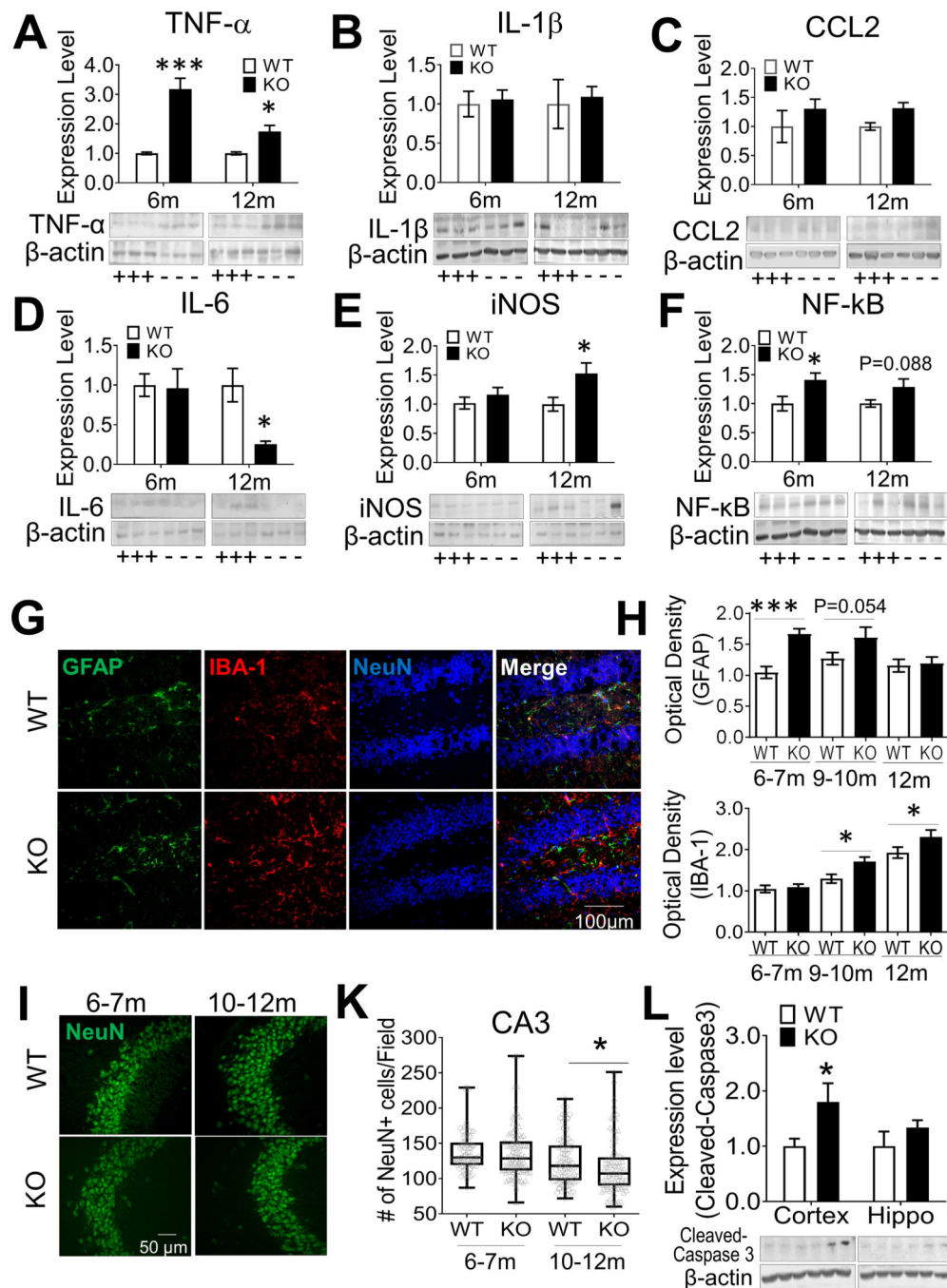
Figure 3. Inflammation and neuronal loss in the GluN3A KO brain.
Chronic inflammation was examined in the hippocampus of WT and GluN3A KO mice of
6–12-month old. Western blot analysis was performed to measure the expression level of
inflammatory factors. **A**-**E.** Quantified results showed significantly increased expression of
TNFα, and iNOS in GluN3A KO mice (marked with "-"), compared to age-matched WT
controls (marked with "+"). Meanwhile, the expression level of CCL2 and IL-1β remained
unchanged and anti-inflammatory IL-6 was largely reduced in the GluN3A KO mice. **F.**
The enhanced expression of NF-κB suggested the involvement of the NF-κB pathway

in the chronic inflammation (n=4 animals in all groups; *P<0.05, **P<0.01, ***P<0.001 vs. WT controls; Two-way ANOVA). **G.** Representative GFAP and IBA-1 staining in the hippocampus of GluN3A KO and WT brains aged at 10–12-month. **H.** Increased numbers of GFAP-positive astrocytes and IBA-1-positive-microglia cells were detected in the hippocampus of GluN3A KO mice, compared to the age-matched controls (n=3 animals in all groups; *P<0.05, ***P<0.001 vs. WT controls; Two-way ANOVA). **I** and **K**. NeuN staining and cell counting were performed to assess neuronal cell loss. At younger ages of 6–7-month old, no significant reduction of neuronal cells was detected in the CA1 and CA3 regions (also see Suppl. Fig. 2B–2C). At the elder age of 10–12-month old, the number of NeuN-positive cells in the CA3 of GluN3A KO mice was significantly less compared to WT mice (n=5 animals in WT group, 7 in KO group; Median ± Interquartile range; *P<0.05 vs. WT, Mann-Whitney U test). **L.** Western blotting for the expression of cleaved-caspase 3 in the hippocampus and cortex of WT and GluN3A KO mice at 10–12-month old. Cleaved caspase-3 was significantly increased in the cortex of GluN3A KO mice (n=8 per group; *P<0.05 vs. WT; Two-way ANOVA). See Suppl. Fig. 5B–5C for the pro-caspase 3 result.
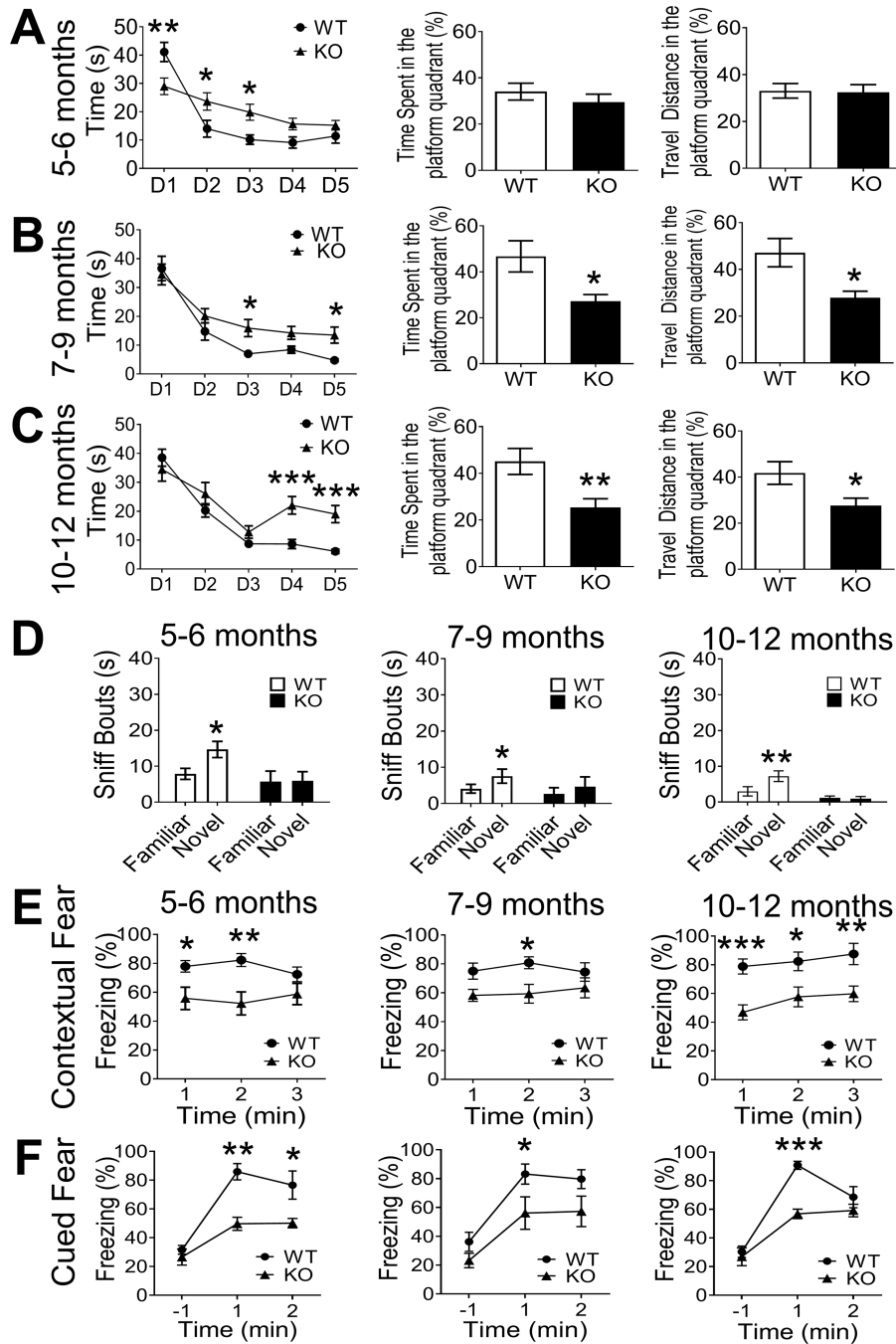
Figure 4. Development of cognition decline in aging GluN3A KO mice

Cognitive function was tested in WT and Glu N3A KO mice at ages of 5 to 12-month old. **A**. Spatial learning and memory ability was tested using the Morris water maze test. At adult ages of 5–6-month old, GluN3A KO mice started to show slower learning ability during the 5 day trial protocol, yet the bar graphs indicates that, one day after the training, GluN3A KO mice did not have difficulties in memorizing the location of the platform. **B**. GluN3A KO mice of 7–9-month old developed slower learning activity during the 5-day training. The time spent and travel distances in the platform quarter of these animals were

significantly less compared to WT mice. **C**. The water maze test was performed in mice at ages of 10–12-month old. GluN3A KO mice suffered significant difficulties in the spatial memory shown as a flat learning curve during the last 3 days of the training period. The bar graphs show marked deteriorations in identifying the platform quarter one day after training compared to WT mice (5–6m: n=12 in WT and n=12 in KO; 7–9m: n=12 in WT and n=9 in KO; 10–12m: n=14 in WT and n=7 in KO; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. WT controls; Two-way ANOVA for training curve and Student's t-test for memory recall test). **D**. In the novel object test, the mouse was allowed to explore 2 identical objects. On the test day, one of the training objects was replaced with a novel object. WT mice sniffed significantly more on the novel object at 1 hr after training, suggesting memory of previous object. GluN3A KO mice showed no preference on these objects (5–6m: n=8 in WT and n=8 in KO; 7–9m: n=8 in WT and n=8 in KO; 10–12m: n=9 in WT and n=8 in KO; *$P<0.05$, **$P<0.01$ vs. familiar object; Two-way ANOVA). **E**. In the context test, the test mouse was reintroduced to the conditioning chamber and recorded for 3 min without stimulation to evaluate the contextual fear. Comparing to age-matched WT mice, GluN3A KO mice aged at 5–9-month old showed significantly less freeze behavior during the first 2 min. Elderly GluN3A KO mice, moreover, had much less freeze behavior during the whole 3-min period. (5–6m: n=11 in WT and n=9 in KO; 7–9m: n=11 in WT and n=13 in KO; 10–12m: n=10 in WT and n=12 in KO; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. WT controls; Two-way ANOVA. The main factor of genetic background (WT vs. KO) is significantly different across the three age groups). **F**. In the cued text, the test mice were introduced to a new chamber with different context background for 3 min, followed by the 30s tone cue (US). The cued fear was accessed by evaluating the freeze behavior after US. GluN3A KO mice aged at 5–12m showed significant less freezing for 2 minutes after US compared to age-matched WT ones. Thus, GluN3A KO mice developed poorer contextual and cued conditioning fear memory (5–6m: n=7 in WT and n=5 in KO; 7–9m: n=8 in WT and n=6 in KO; 10–12m: n=4 in WT and n=9 in KO; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. WT controls; Two-way ANOVA and main factor of genetic background (WT vs. KO) is significantly different across the three age groups).
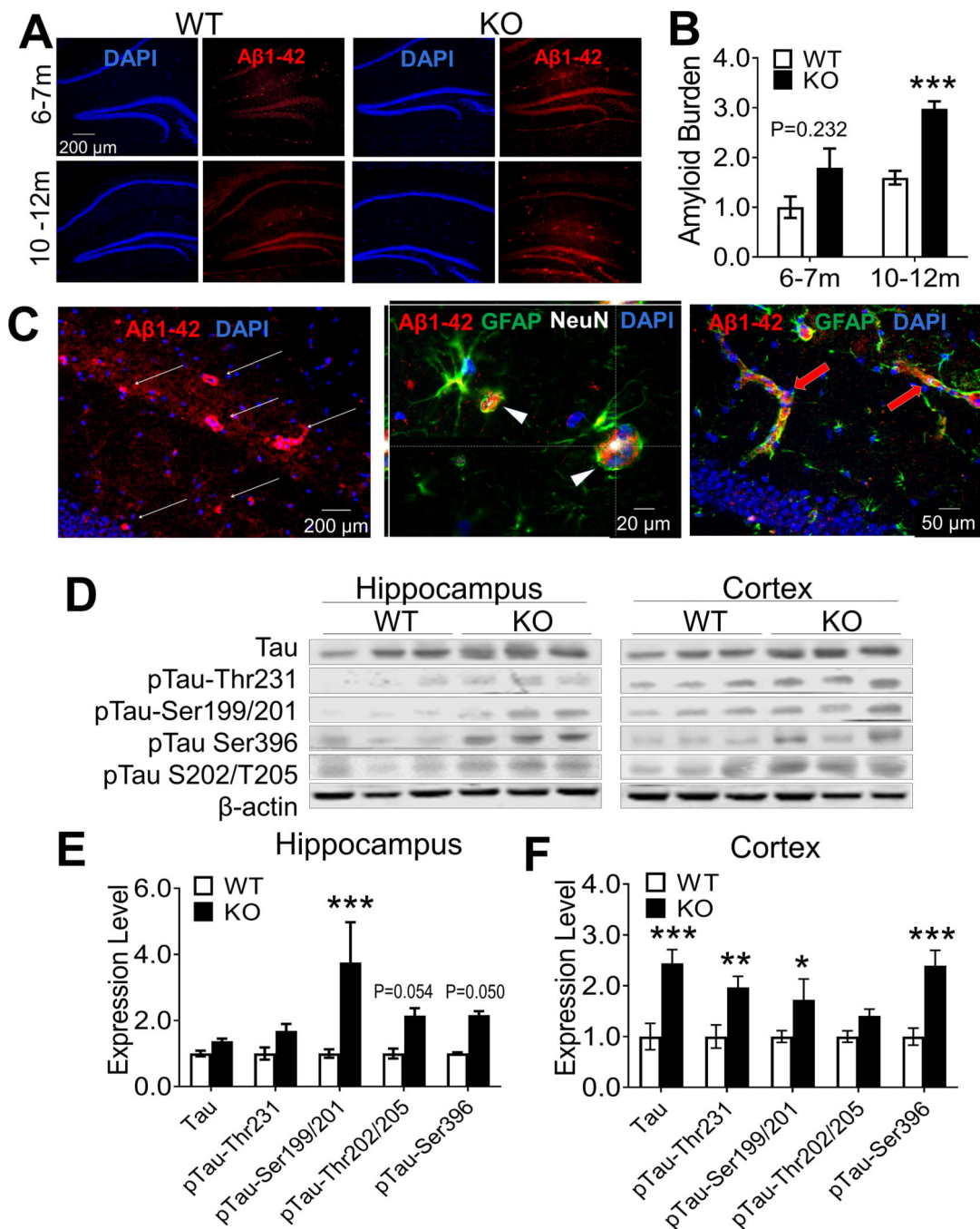
Figure 5. Amyloid and tau pathology in the aging GluN3A KO brain

Aβ deposition and tau protein phosphorylation were examined in WT and GluN3A KO brains of 3 to 12 months old. **A**. Immunohistochemical staining using the amyloid antibody against the 1–42 peptide revealed Aβ depositions in the CA1 and DG of GluN3A KO mice of 6–12-month old (for negative result in the 3-month old brain, see Suppl. Fig. 5A). **B**. Significant Aβ accumulations were detected in the GluN3A KO brain of 10–12 months old (n=5 in WT and 6 in KO group; ***$P<0.001$ vs. WT; Two-way ANOVA). **C**. Images from brain section staining of female GluN3A KO mice of 15-months old. Deposition of

Aβ 1–42 was seen in the hippocampus of GluN3A KO mice. The middle 3-D image shows co-labeling of Aβ (red) and NeuN (white) in the same cells, suggestive of intracellular deposition of Aβ in neuronal cells (arrowhead). Hypertrophic reactive astrocytes positive to GFAP staining (green) are frequently seen adjacent to Aβ-positive cells as seen in AD brains. The yellow color of GFAP-positive cells (green) also indicates Aβ deposition in astrocytes. The image on right shows Aβ distribution along vessel-like structures (red arrows). **D**. Western blot was applied to measure tau phosphorylation, including changes of some common phosphorylation sites of the tau protein. **E** and **F**. Summarized Western blot measurements of tau protein and phosphorylated forms in 10–12-month old mice. The phosphorylation at several common sites was significantly increased or tentatively increased in the hippocampus of GluN3A KO mice compared to levels in WT. Phosphorylation levels of most tau proteins tested was significantly increased in the cortex of GluN3A KO mice (n=6 per group; *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. WT; Two-way ANOVA and the main factor of genetic background (WT vs. KO) is significantly different in both hippocampus and cortex (E-F, $P<0.001$). For analysis of the ratio of p-tau against total proteins see Suppl. Fig. 6E–6F.
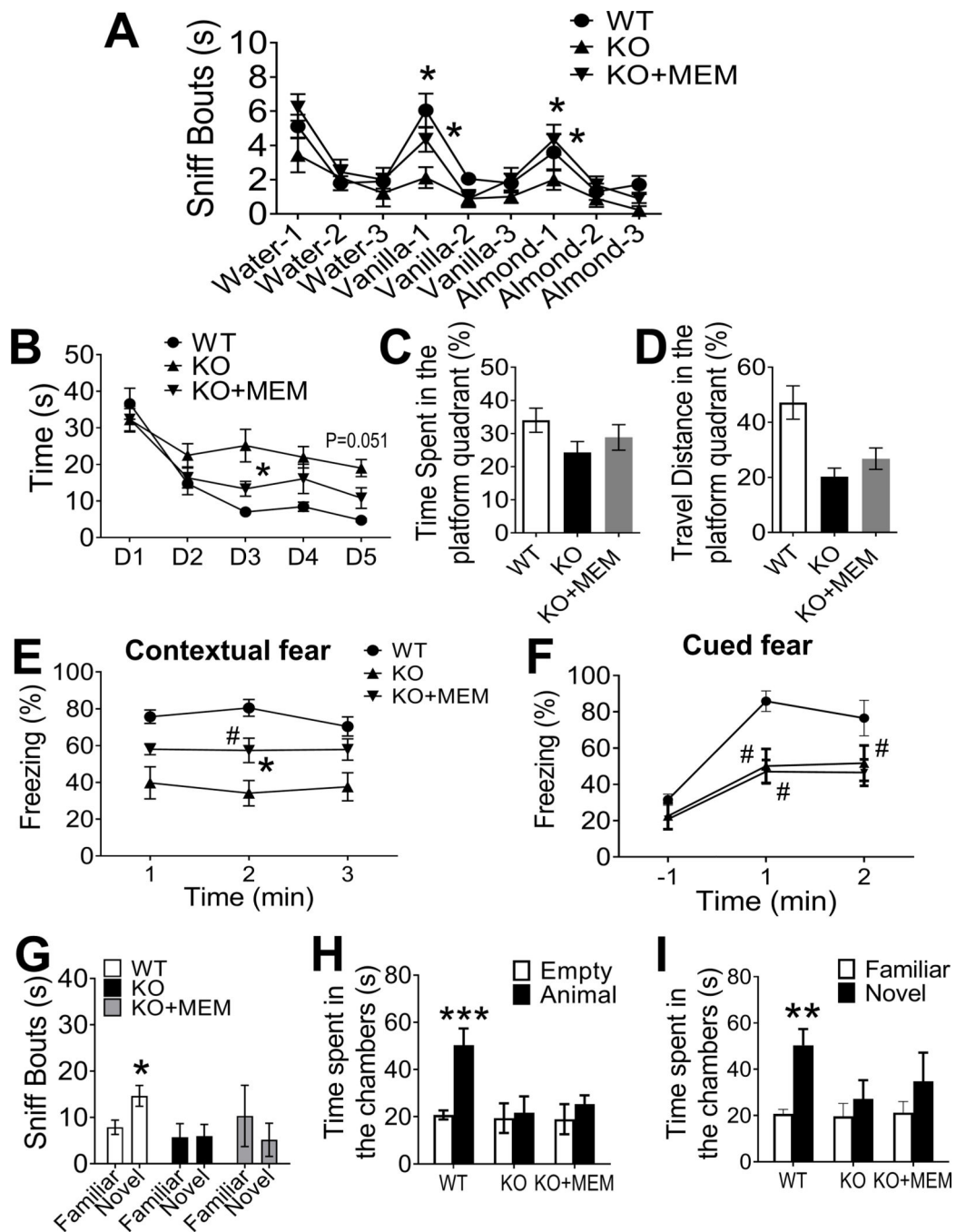
Figure 6. Early and daily memantine treatment alleviated the development of AD-associated behaviors in GluN3A KO mice

To test effects of daily chronic MEM treatments in GluN3A KO mice, MEM (10 mg/kg/day in drinking water) or vehicle control (regular water) was started from the preclinical stage of 3-month old when olfactory dysfunction had emerged but no detectable cognitive deficits. Functional and behavioral tests were performed after the daily treatment for 3 months.

A. In the olfactory habituation/dishabituation test, the vehicle group of GluN3A KO mice continued to be insensitivity to the initial exposure to vanilla and almond. The MEM group,

however, showed significant improvements in this test; the treatment basically reversed the functional deficit to the WT control level (WT: n=19, KO: n=9, KO+MEM: n=9; *P<0.05 KO+MEM vs. KO; One-way ANOVA). **B**. During the 5-day training of the water maze test, the chronic 3-month MEM treatment significantly improved the learning curve closer to WT control (WT: n=7, KO: n=8, KO+MEM: n=9; *P<0.05, KO+MEM vs. KO. Two-way ANOVA; the main factor between KO control and treatment groups showed significant difference *(P<0.001). **C** and **D**. One day after the 5-day training period, GluN3A KO mice received the MEM treatment appeared to perform better (more time and travel distance in the platform quadrant), but the change was not statistically significant (same experimental groups as in B; One-way ANOVA). **E** and **F**. In the fear conditioning memory test, WT mice exhibited high rate (~80%) of freeze reaction due to normal contextual memory while GluN3A KO mice exhibited a low (~40%) freeze response as a result from losing the memory. The 3-month MEM treatment significantly improved the contextual memory of GluN3A KO mice, demonstrated by a significantly higher rate (~60%) of freeze behavior. Meanwhile, the cued memory was not improved in the GluN3A mice with MEM treatment (WT: n=10, KO: n=8, KO+MEM: n=8; *P<0.05 KO+MEM vs. KO; #P<0.05 KO+MEM vs. WT; Two-way ANOVA). **G.** MEM did not improve visual/recognition memory of GluN3A KO mice in the novel object test (WT: n=8, KO: n=8, KO+MEM: n=8). **H** and **I**. In the three-chamber test, the MEM treatment had no effect on the sociability of GluN3A KO mice (G); there appeared a trend of improvement in social novelty behavior with no statistical significance (H) (WT: n=9, KO: n=8, KO+MEM: n=9; ***P<0.001, animal vs. empty chamber, **P<0.01 Novel vs. Familiar object; Two-way ANOVA).

Table 1.

Basal intracellular Ca^{2+} concentration in WT and GluN3A KO neurons

Brain region	Age	WT $[Ca^{2+}]_i$ (nM)	N	GluN3A KO $[Ca^{2+}]_i$ (nM)	N	*P* value
CA1	6–7 month	71.6±39.3	51 cells/4 mice	133.3±31.5	51 cells/5 mice	0.09
	10–12 month	61.7±26.1	53 cells/4 mice	107.6±24.5	57 cells/4 mice	0.19
DG	6–7 month	72.2±31.2	51 cells/4 mice	153.0±43.1	51 cells/5 mice	0.12
	10–12 month	62.4±24.9	53 cells/4 mice	109.7±24.3	57 cells/4 mice	0.17

The unpaired Student *t* test was performed in statistical analysis.