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Early Fear Memory Defects Are Associated with Altered Synaptic Plasticity and Molecular Architecture in the TgCRND8 Alzheimer's Disease Mouse Model

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Abstract

Alzheimer's disease (AD) is a complex and slowly progressing dementing disorder that results in neuronal and synaptic loss, deposition in brain of aberrantly folded proteins, and impairment of spatial and episodic memory. Most studies of mouse models of AD have employed analyses of cognitive status and assessment of amyloid burden, gliosis, and molecular pathology during disease progression. Here, we sought to understand the behavioral, cellular, ultrastructural, and

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molecular changes that occur at a pathological stage equivalent to early stages of human AD. We studied the TgCRND8 mouse, a model of aggressive AD amyloidosis, at an early stage of plaque pathology (3 months of age) in comparison to their wild-type littermates and assessed changes in cognition, neuron and spine structure, and expression of synaptic glutamate receptor proteins. We found that, at this age, TgCRND8 mice display substantial plaque deposition in the neocortex and hippocampus and impairment on cued and contextual memory tasks. Of particular interest, we also observed a significant decrease in the number of neurons in the hippocampus. Furthermore, analysis of CA1 neurons revealed significant changes in apical and basal dendritic spine types, as well as altered expression of GluN1 and GluA2 receptors. This change in molecular architecture within the hippocampus may reflect a rising representation of inherently less stable thin spine populations, which can cause cognitive decline. These changes, taken together with toxic insults from amyloid- β protein, may underlie the observed neuronal loss.

Indexing Terms

Amyloid beta; mouse model of dementia; neuronal morphology; dendritic pathology; spine pathology

Introduction

Alzheimer's disease (AD) is a complex, and slowly progressive form of age-related dementia that ultimately results in major deficits in spatial and episodic memory, following the accumulation of toxic amyloid- β protein (A β) species (Gandy, 2012; Gandy and DeKosky, 2013). The neuropathological hallmarks of AD develop over the course of 2-3 decades and include extracellular accumulation of A β , intracellular accumulation of hyperphosphorylated microtubule-associated protein tau, synaptic degeneration, and neuronal loss (for review see Nelson et al., 2012). One of the earliest hallmarks of AD is synaptic degeneration, which likely accounts for the subtle cognitive defects at early stages of the disease (DeKosky and Scheff, 1990; Scheff et al., 1990; Akram et al., 2008).

Recent studies indicate that accumulation of soluble oligomeric assemblies of A β (oA β), but not insoluble A β plaques, is closely associated with the appearance of memory defects in some animal models of AD (Lefterov et al., 2009; Gandy et al., 2010). Moreover, low-n oA β assemblies accumulate within neurons at synaptic sites, alter the regulation of synaptic plasticity, and may affect memory (Capetillo-Zarate et al., 2012). These subtle defects in synaptic plasticity have been reported prior to onset of plaque pathology in the Tg2576 mouse model of AD, suggesting that early disease processes can be studied in AD mouse models that harbor familial early-onset AD (FAD)-associated mutations (Jacobsen et al., 2006; Ashe and Zahs, 2010). Studying AD mouse models at early stages of A β pathology and cognitive defect provides insight into the cellular and molecular changes that occur at onset of clinical symptoms, whereas the exclusive study of late-stage pathology in the brains of older mice may be comparable to late stages of human AD that are less likely to respond to therapy (Gandy, 2011).

We studied early synaptic and neuronal changes in the TgCRND8 mouse model of AD. These mice harbor FAD mutations within the human amyloid precursor protein (APP) 695

isoform (Swedish K670M/N671L and Indiana V717F mutations) under control of the hamster prion promoter, overexpress human A β by 3–4 months, and at 6 months have a high A β_{42} /A β_{40} ratio and severe plaque deposition (Chishti et al., 2001). Previous studies indicate that by 3 months of age, TgCRND8 mice are impaired on acquisition and learning reversal in the reference memory version of the Morris water maze (MWM) (Janus et al., 2000; Chishti et al., 2001; Hyde et al., 2005) and exhibit synaptic overexcitability in the hippocampus at 5 months (Chishti et al., 2001). At this age, TgCRND8 mice have begun to deposit A β plaques throughout the neocortex and hippocampus, features of neuropathology that are comparable to early-stage AD in humans (Hyde et al., 2005; Hanna et al., 2012).

In this study, we assessed morphological changes in neurons, spines and synapses in early stages of the disease and correlated them to cognitive impairment and A β load. We report that 3-month-old TgCRND8 display defective cued and contextual fear memory, which parallels alterations in dendritic spine morphology and molecular architecture, but without changes in neuronal morphology. Using a technique to estimate total cell and total neuronal numbers, we also noted a reduction in number of neurons in the hippocampus of 3-month-old TgCRND8 mice by comparison to their wild-type (wt) littermates. Taken together with prior studies (Janus et al., 2000; Hyde et al., 2005; Yang et al., 2011; Hanna et al., 2012; Steele et al., 2013a; Steele et al., 2013b) our data suggest that the early alterations in synaptic stability and neuronal homeostasis are associated with A β accumulation and memory failure in the TgCRND8 AD mouse model.

Materials and Methods

Experimental animals

All mice used in the current study were 3-month-old males, group-housed, given *ad libitum* access to food and water, and housed in micro-isolator cages under a 12-hour light/dark cycle. For behavioral assessments, we used 19 TgCRND8 and 18 wt mice; for Western blot analysis, 5 mice/group; for isotropic fractionator cell count determination, 7 TgCRND8 and 8 wt; 5 TgCRND8 and 5 wt for cell loading with a minimum of 5 neurons/mouse and 5 TgCRND8 and 5 wt for electron microscopy (EM) experiments. All animal procedures were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Experimental Animals and were approved by the Institutional Animal Care and Use Committee at the Icahn School of Medicine at Mount Sinai.

Behavioral testing

Mice were tested for cued and contextual fear memory as previously described (Jacobsen et al., 2006; Yang et al., 2011; Steele et al., 2012). Briefly, mice were trained and tested in operant chambers on three consecutive days in the cued and contextual fear conditioning paradigm. On Day 1, mice were placed into Context A (black/white checked walls, grid floor, houselights at 100%) and allowed to explore for 120 s (baseline) prior to three 30-s tone/shock pairings (30-s, 4-kHz pure tone co-terminating with a 2-s scrambled 0.6-mA foot-shock). Each tone/shock pairing was separated by 30 s of exploration time and animals were given 30 s to explore following the final tone/shock pairing (300 s total). On Day 2, mice were placed into Context B (gray walls, black plastic floor, houselights at 50%) and

allowed to explore for 180 s in the constant presence of the 4-kHz pure tone. On Day 3, mice were replaced into Context A and allowed to explore for 180 s without the tone. Freezing was defined as a lack of movement except that required for respiration. Memory for the context (contextual memory) or the tone (cued memory) for each animal was obtained by subtracting the percent freezing during baseline from the percent freezing on day 2 or day 3, respectively. Freezing behavior was recorded remotely and analyzed using Stoelting ANY-MAZE Fear Conditioning Software (Stoelting, Wood Dale, IL).

Antibodies

Details regarding each of the primary antibodies used in this study are summarized in Table 1.

Polyclonal antibody 369 recognizes the C-terminus of β APP⁶⁴⁵⁻⁶⁹⁴ (VPAVPAVSLVPPAFVSMPPVPPPGFNPIPPPPFLRASFNPSQPPPGFMP; amino acids correspond to those of human β APP₆₉₅). Specificity was shown with Western blot analysis, which resulted in approximate reactivity of a protein with a molecular weight of 12-16 kDa (C-terminal fragments) and 100-130 kDa (immature and mature full-length APP) as previously described (Gandy et al., 1988; Buxbaum et al., 1990). We have also demonstrated similar results (Gandy et al., 2010; Steele et al., 2013b).

Monoclonal antibody 1G6 recognizes the cleaved C-terminus of A β ₄₂. We and others, have shown that this antibody specifically stains A β plaques in the brains of AD model transgenic mice that overexpress mutated forms of APP (Parvathy et al., 2001; Steele et al., 2013b).

Monoclonal antibody 6E10 (Covance, Princeton, NJ) recognizes amino acids 1-16 of human A β , with the epitope lying within amino acids 3-8 of A β (EFRHDS). We have previously shown that this antibody reliably labels amyloid plaques in the brains of AD transgenic mice and well as APP and A β protein in Western blot and ELISA (Brautigam et al., 2012; Steele et al., 2013b).

The anti- β -actin monoclonal antibody (Sigma, St Louis, MO) was derived from the AC-15 hybridoma and recognizes an epitope located in the N-terminal end of the β -isoform of actin. Specificity was shown through immunoblotting which generated the expected 42 kDa molecular weight protein (Gimona et al., 1994; North et al., 1994) as well as with immunofluorescence of cultured cells and immunohistochemistry of smooth muscle from chicken gizzards (North et al., 1994).

The anti-NeuN monoclonal antibody (Millipore, Billerica, MA) specifically recognizes the DNA-binding, neuron-specific protein NeuN. Only one monoclonal antibody exists whose specificity was shown by immunohistochemistry with the antibody only reacting with neuronal cell bodies and not GFAP-positive glial cells (Mullen et al., 1992). Western blot analysis also results in two to three bands in the 46-48 kDa range that is specific to nuclei of postmitotic neurons only (Mullen et al., 1992; Luuk et al., 2008).

The anti-GluN1 polyclonal antibody (Millipore) recognizes the C-terminus of Rat GluN1 splice variant isoform D. The specificity was determined in Western blot and

immunoprecipitation of primary neuronal cultures with transfected with recombinant GluN1 proteins (Tingley et al., 1993).

The anti-GluA2 polyclonal antibody (Millipore) recognizes the C-terminus of rat GluA2. Specificity was shown previously in Western blots in which transfected cells showed that the antibody was selective for GluA2 reactivity (single band with 108 kDa molecular weight) with no cross-reactivity to GluA1, GluA3, or GluA4 (Wong-Riley and Jacobs, 2002; Van Damme et al., 2003).

Tissue preparation

Following behavioral testing, animals were sacrificed and transcardially perfused with ice-cold phosphate buffered saline (PBS; pH 7.4). Brains were removed and dissected into hippocampus, neocortex, and cerebellum, snap-frozen and stored at -80°C for biochemical analysis. Snap-frozen tissue was homogenized as described in (Steele et al., 2013b). Briefly, tissue was dissociated by 20 up-and-down strokes of a glass-Teflon homogenizer at 500 rpm in 1% Triton X-100/TBS (pH 7.6) with protease/phosphatase inhibitors [1 mM EDTA, 1 mM Na₃VO₄, 5 μM ZnCl₂, 100 mM NaF, 1 μM pepstatin, 1 mM PMSF, mini-complete protease inhibitor tablet (Roche, Indianapolis, IN)]. The 1% Triton X-100 homogenate was then ultracentrifuged at 100,000 g for 1 hour at 4°C and the supernatant was removed, aliquoted, and stored at -80°C until analysis. The 1% Triton X-100-insoluble pellet was then homogenized in 70% formic acid by 20 up-and-down strokes of a glass-Teflon homogenizer at 500 rpm, ultracentrifuged as described above, and the liquid phase was removed, neutralized by 1:20 dilution into 1 M Tris (pH 11.0), aliquoted, and stored at -80°C until analysis. All homogenization and sample collection steps were performed on ice and samples were aliquoted to avoid denaturing of protein by repeated freeze-thaw cycles. The Triton X-100-soluble fraction was analyzed by Western blot for levels of APP metabolites. The Triton X-100-insoluble fraction (70% formic acid-soluble fraction) was analyzed for Aβ₄₂ content by sandwich ELISA as an indicator of “insoluble” Aβ₄₂ content, which typically comprises fibrillar and larger aggregates, as well as insoluble monomeric Aβ₄₂ (Steele et al., 2013b; Lublin and Gandy, 2010; Gandy et al., 2010).

Western blot analysis

Following BCA protein estimation assay (Pierce, Rockford, IL), brain homogenates were prepared for SDS-PAGE analysis. For Western blot analysis, 20-75 μg total protein was denatured under reducing conditions in sample loading buffer [20 mM Tris, 1% glycerol, 180 mM β-mercaptoethanol, 0.003% bromophenol blue, and 2% SDS, pH 6.8] by boiling for 10 min at 100°C prior to loading onto a 12% Bis-Tris (Bio-Rad, Hercules, CA) gel for SDS-PAGE with NuPAGE MES buffer (Invitrogen, Grand Island, NY), then transferred to PVDF membrane (0.22 μm; Millipore) using the Criterion Blotter system (Bio-Rad) equipped with plate electrodes. Following SDS-PAGE, membranes were blocked for 1 hr at room temperature in 5% w/v non-fat milk (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS containing 0.1% v/v Tween-20 (Fisher Scientific, Pittsburgh, PA; TBS-T). Membranes were then incubated in the indicated primary antibody (in 5% milk/TBS-T) overnight at 4°C, washed 4 times in TBS-T, incubated in species-specific HRP-conjugated secondary antibody (in 5% milk/TBS-T) for 1 hour at room temperature, and then washed 4 times in TBS-T.

Membranes were subsequently developed with ECL Western blotting substrate (Pierce) using the Fujifilm LAS-3000 developer. Membranes were then washed once in TBS-T and stripped in low pH stripping buffer (25 mM glycine-HCl, pH 2.0 and 1% w/v SDS) with vigorous shaking to remove primary and secondary antibody, washed 3 times in TBS-T, and blocked for 1 hour (in 5% milk/TBS-T) at room temperature before probing with the next primary antibody.

Fluorescence immunohistochemistry

Immunostaining with mAb anti-A β ₄₂-C-terminus Pan1G6 was performed as described in Steele et al. (2012). Briefly, hemispheres were postfixed in 4% (w/v) paraformaldehyde in PBS (pH 7.4) and dehydrated via serial equilibration into 10%, 20%, and then 30% sucrose/PBS, then sectioned coronally at 30 μ m thickness using a Leica CM3050 cryostat. Series of every 5 sections were stained from each animal. On the first day, sections were washed in PBS (pH 7.4) prior to 20 min antigen retrieval in 80% formic acid. Sections were subsequently washed 3 $\times$ 10 min and blocked in 3% goat serum (Gibco, Grand Island, NY) in PBS (pH 7.4) containing 0.25% (v/v) Triton X-100 (Sigma). Sections were then incubated overnight at 4°C in mouse mAb Pan1G6 (1:500) in 1% goat serum/PBS. On the second day, sections were washed 6 $\times$ 30 min and incubated overnight at 4°C in goat anti-mouse-AlexaFluor 488 (1:500) in 1% goat serum/PBS. On the following day, sections were again washed 6 $\times$ 30 min in PBS, mounted onto slides, and coverslipped using Vectashield Hardest Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) and allowed to cure overnight. Sections were then imaged using an Olympus Bx61 microscope (Center Valley, PA).

Sandwich ELISAs and duplicate epitope immunoassay

Samples were diluted into ELISA capture buffer to within linear range and were analyzed for concentration of human A β ₄₂ monomer by sandwich ELISA according to manufacturer protocol (Wako, Richmond VA). Absolute A β ₄₂ concentration was corrected for dilution factor and then normalized to tissue weight prior to statistical analysis (i.e., pg/g = [analyte]/tissue weight).

Isotropic fractionator

The isotropic fractionator is a fast and reliable technique for determining total cell and neuronal counts, as well as cell densities in the brain (Herculano-Houzel and Lent, 2005; Brautigam et al., 2012). It involves processing of highly anisotropic brain structures into homogeneous isotropic suspensions of cell nuclei that can be easily quantified (Herculano-Houzel and Lent, 2005). Because the estimates of cell counts are obtained separately from the determination of brain volume, the two measurements can be used in comparative studies of brain-volume variation (i.e., comparing across species or between different mouse lines; (Herculano-Houzel et al., 2006). Dissected brain regions were prepared and analyzed as described in (Brautigam et al., 2012). To visualize nuclei and determine total cell counts, cell slurries were incubated with DAPI; to determine total neuron counts, cell slurries were incubated with anti-NeuN monoclonal antibody (Millipore; 1:200) for 2 hours followed by incubation in a secondary anti-mouse IgG-Alexa-Fluor 594 for 1.5 hours (Millipore; 1:200 in PBS), as described previously (Brautigam et al., 2012). Cells were then counted with a

hemocytometer using a 20X/0.45 N.A., Plan-Apochromat objective on a Zeiss Axiophot 2 microscope equipped with a motorized stage. Both the upper and lower grids of the hemocytometer and all four quadrants were counted and averaged. Total cell numbers and total neuronal numbers were calculated by multiplying the number of cells/ml by final volume. Densities were calculated by dividing the number of cells/ml by the mass of brain region.

Intracellular dye injections

Mice were anesthetized and perfused with 1% PFA followed by 4% PFA with 0.125% glutaraldehyde as described previously (Dickstein et al., 2010). The brains were removed, hemisected, and postfixed overnight, sectioned on a Vibratome VT1000S (Leica, Buffalo Grove, IL) into 200 μ m-thick sections and stored at 4 °C in PBS until ready for use. For intracellular injections, sections were incubated in DAPI for 5 min to reveal the cytoarchitectural features of the pyramidal layer of CA1 of the hippocampus. The sections were then mounted on nitrocellulose paper and immersed in ice-cold 0.1 M PBS. Pyramidal neurons in the dorsal CA1 region of the hippocampus were injected with an intracellular iontophoretic injection of 5% Lucifer Yellow (Molecular Probes, Grand Island, NY) in distilled water under a DC current of 3-8 nA until dye had completely filled distal processes (Radley et al., 2006; Radley et al., 2008; Dickstein et al., 2010; Tyan et al., 2012). Five to 10 neurons were injected per slice and placed far enough apart to avoid overlapping of their dendritic trees. Brain sections were then mounted on gelatin-coated glass slides and cover slipped in Fluoromount G slide mounting media (Cell Lab Beckman Coulter, Fullerton, CA).

Neuronal and dendritic reconstruction

In order for a loaded neuron to be included in the analysis, it had to satisfy the following criteria: (1) reside within the pyramidal layer of the CA1 as defined by cytoarchitectural characteristics; (2) demonstrate complete filling of dendritic tree, as evidenced by well-defined endings; and (3) demonstrate intact tertiary branches, with the exception of branches that extended beyond 50 μ m in radial distance from the cell soma (Radley et al., 2006; Radley et al., 2008; Dickstein et al., 2010; Midthune et al., 2012; Tyan et al., 2012). Neurons meeting these criteria were reconstructed in 3-dimension (3D) with a 40x/1.4 N.A., Plan-Apochromat oil immersion objective on a Zeiss Axiophot 2 microscope equipped with a motorized stage, video camera system, and Neurolucida morphometry software (MBF Bioscience, Williston, VT). Using NeuroExplorer software (MBF Bioscience) total dendritic length, number of intersections, and the amount of dendritic material per radial distance from the soma, in 30- μ m increments (Sholl, 1953) were analyzed in order to assess morphological cellular diversity and potential differences among animal groups.

Confocal microscopy and analysis of dendritic spines

Using an approach that precludes sampling bias of spines, CA1 dendritic segments were selected with a systematic-random method (Radley et al., 2006; Radley et al., 2008). Dendritic segments were acquired as previously described (Bloss et al., 2011; Midthune et al., 2012; Tyan et al., 2012). Briefly, 20-25 μ m in length, were imaged on a Zeiss CLSM 510 microscope (Zeiss, Thornwood, NY) using a 100x/1.4 N.A. Plan-Apochromat objective

with a digital zoom of 3.5 and an Ar/Kr laser at an excitation wave length of 488 nm. All confocal stacks were acquired at 512×512 pixel resolution with a z-step of 0.1 μ m, a pinhole setting of 1 Airy unit and optimal settings for gain and offset. All confocal stacks included approximately 1 μ m above and below the identified dendritic segment. On average 6 z-stacks were imaged per neuron, 3 for the apical dendritic tree and 3 for the basal dendritic trees. All dendritic segments had to be either parallel or at acute angles to the coronal surface of the section, and could not overlap other segments that would obscure visualization of spines (Radley et al., 2006; Radley et al., 2008). Confocal stacks were then deconvolved using an iterative blind deconvolution algorithm (AutoDeblur version 8.0.2; MediaCybernetics, Rockville, MD).

After deconvolution, confocal stacks were analyzed with NeuronStudio (Wearne et al., 2005; Rodriguez et al., 2006; Rodriguez et al., 2008; Rodriguez et al., 2009) (<http://www.mssm.edu/cnic>) to examine global and local morphometric characteristics of dendrites and spines such as dendritic spine densities and dendritic spine shape (stubby, thin and mushroom). This software allows for automated digitization and reconstructions of neuronal morphology in 3D from multiple confocal stacks on a spatial scale and averts the subjective errors encountered during manual tracing using a Rayburst-based spine analysis (Wearne et al., 2005; Rodriguez et al., 2006; Rodriguez et al., 2008; Rodriguez et al., 2009). At least five neurons per mouse and three apical and three basal dendritic segments per neuron were analyzed with each segment manually inspected and appropriate corrections such as mislabeled spines due to background artifacts, made using the NeuronStudio interface.

Dendritic spines along each segment were analyzed for evidence of spatial clustering, which may be controlled actively in order to enhance the crosstalk between and nonlinear summation of incoming signals on nearby spines (Harvey and Svoboda, 2007; De Roo et al., 2008; Larkum and Nevian, 2008; Takahashi et al., 2012). Spatial clusters of three or more spines along each dendritic segment were detected with our recent automated algorithm (Yadav et al., 2012), which uses the traversed distance along the dendritic segment as a one-dimensional distance metric and is based on the clustering algorithm known as unweighted pair group method with arithmetic mean (UPGMA; Yadav et al., 2012). The algorithm reports the C-score, an analog to statistical p-values, which quantifies the probability that the number of clustered spines occurred due to random placement alone. (In randomly clustered data, the distribution of C-scores cannot be distinguished from a normal distribution.) The C-score of each dendrite segment was approximated with Monte Carlo simulations, which randomized placement of spines along 5,000 digital copies of the original branch and then repeated the clustering algorithm on each copy. For each branch analyzed, data reported include the C-score, the percentage of spines that were identified as clustered, and the proportion of stubby, mushroom, and thin spines among the clustered spines.

Electron microscopy and synapse density analysis

The opposite hemispheres from the cell loading experiments above were used for electron microscopy (EM) analyses. Coronal sections (250 μ m-thick) encompassing the dorsal CA1 region of the hippocampus were prepared for as reported by Janssen and colleagues (2005). Briefly, slices were cryoprotected in graded phosphate buffer/glycerol washes at 4°C, and

manually microdissected to obtain blocks containing the CA1 region. The blocks were rapidly freeze-plunged into liquid propane cooled by liquid nitrogen (-190°C) in a Universal Cryofixation System KF80 (Reichert-Jung, Depew, NY) and subsequently immersed in 1.5% uranyl acetate dissolved in anhydrous methanol at -90°C for 24 hours in a cryosubstitution unit (Leica). Block temperatures were raised from -90 to -45°C in steps of 4°C/hour, washed with anhydrous methanol, and infiltrated with Lowicryl resin (Electron Microscopy Sciences, Hatfield, PA) at -45°C. The resin was polymerized by exposure to ultraviolet light (360 nm) for 48 hours at -45°C followed by 24 hours at 0°C. Block faces were trimmed and ultrathin sections (90 nm) were cut with a diamond knife (Diatome, Hatfield, PA) on an ultramicrotome (Reichert-Jung) and serial sections of at least 5 sections were collected on formvar/carbon-coated nickel slot grids (Electron Microscopy Sciences).

For synapse quantification, serial section micrographs were imaged at 12,000x on a Hitachi H-7000 transmission electron microscope using an AMT Advantage CCD camera (Advanced Microscopy Techniques). Fifteen sets of serial images across the same set of 5 consecutive ultrathin sections were taken in the stratum radiatum of the CA1 field and imported into Adobe Photoshop (version CS5, Adobe Systems). To obtain a stereologically unbiased population of synapses for quantitative morphologic analysis, we used a disector approach on ultrathin sections (Dumitriu et al., 2010; Hara et al., 2011). Briefly, all axospinous synapses were identified within the first 2 and last 2 images of each 5-section serial set, and counted if they were contained in the reference image but not in the corresponding look-up image. To increase sampling efficiency, the reference image and look-up image were then reversed; thus each animal included in the current study contributed synapse density data from a total of 30 disector pairs. The disector area used was 86.1184 μm^2 , and the height of the disector was 180 nm. Axospinous synapse density (per μm^3) was calculated as the total number of counted synapses from both images divided by the total volume of the disector. The criteria for inclusion as an axospinous synapse included the presence of synaptic vesicles in the presynaptic terminal and a distinct asymmetric postsynaptic density (PSD) separated by a clear synaptic cleft. The synapse density was calculated as the total number of counted synapses divided by the total volume of the disectors used. For synapse to be scored as perforated it had two or more separate PSD plates.

Statistics

All statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA). For neuron morphology and spine and synapse data, values from single cells were averaged, and then averaged for each animal. These values were used for comparison of means. For the mean apical and basal dendritic length, spine and synapse data, behavior analysis, isotropic fractionator, and Western blot analysis: independent samples Student's *t* tests were performed comparing TgCRND8 mice to their littermate controls. Sholl analyses (dendritic length from soma and number of intersections) were analyzed using two-way repeated measures ANOVA with genotype as a between-groups factor and radial distance from soma (in 30- μm increments) as a within-group factor (Midthune et al., 2012; Tyan et al., 2012). Differences at individual distances in the Sholl analysis were determined with Bonferroni *post hoc* tests. Determination of changes in

dendritic spine types was analyzed using two-way ANOVA with genotype as a between-groups factor and spine type as a within-group factor followed by Bonferroni's *post hoc* tests. Spine clustering C-score distributions were compared with the Kolmogorov-Smirnov test. In all instances, Shapiro-Wilk test of normality and Levene's test for homogeneity of variance were used for inclusion in parametric tests ($p > 0.05$ for Shapiro-Wilk and Levene's tests). Significance for all tests is reported with $p < 0.05$ using two-tailed tests with an α level of 0.05.

Results

TgCRND8 mice display impaired cued and contextual memory at early stage A β pathology

To assess whether there was a connection between cognitive deficit and molecular, synaptic, and neuroanatomical pathology at this early stage, we tested TgCRND8 mice using a contextual and cued fear memory paradigm. We found that, at 3 months of age, TgCRND8 mice exhibited significantly less contextual memory ($t_{(35)} = 4.211$; $p = 0.0002$), as well as cued memory ($t_{(35)} = 5.169$; $p < 0.0001$), by comparison to their non-transgenic littermates (Fig. 1C). These results indicate that, in addition to the previously published impairment on the MWM (Chishti et al., 2001; Hyde et al., 2005), TgCRND8 mice are also impaired on both contextual and cued fear memory tasks at this stage of plaque pathology.

TgCRND8 mice display plaque pathology and altered A β 42 concentrations

We next examined the amyloid pathology present in 3-month-old TgCRND8 mice. Using PanIG6 immunoreactivity, TgCRND8 mice have definite plaque deposition throughout the hippocampus and neocortex compared to wt mice (Fig. 1A). Moreover, ELISA assays specific for insoluble A β 42, using a human APP-specific anti-A β 42 sandwich ELISA, found that absolute A β 42 concentration in the hippocampus (893,931 pg/g; SD = 80,770) of TgCRND8 mice were consistent with plaque pathology (Fig 1A). A β 42 was expectedly below the limit of detection in samples derived from wt mice.

Decrease in number of hippocampal neurons among TgCRND8 mice

Using the isotropic fractionator, we recently reported significant neuronal loss in the hippocampus of 6-month old TgCRND8 mice (Brautigam et al., 2012). Using the same method, we observed significant neuronal loss only in the hippocampus of TgCRND8 mice compared to controls ($t_{(8)} = 2.506$; $p = 0.034$), but without neuronal loss in the neocortex and cerebellum (Fig. 2). Moreover, no changes in total cell numbers (i.e., neurons and glial cells) were observed in the neocortex, hippocampus, or cerebellum.

Normal dendritic length and arborization of CA1 pyramidal neurons in TgCRND8 mice

In the present study, we examined changes in CA1 pyramidal neurons in 3-month-old TgCRND8 AD model mice. A total of 25 TgCRND8 and 29 wt cells met morphological criteria for inclusion in this study. Representative examples of CA1 dendritic arbor reconstructions and dendrograms are depicted in Figure 3A,B. Surprisingly, we found that compared to wt, CA1 pyramidal neurons from transgenic mice do not exhibit any significant alteration in total apical dendritic length (wt = $2502 \pm 187 \mu\text{m}$; TgCRND8 = $2514 \pm 260 \mu\text{m}$) or mean basal dendritic length (wt = $444 \pm 27 \mu\text{m}$; TgCRND8 = $427 \pm 25 \mu\text{m}$) at this early

stage of plaque pathology (Fig. 3C,D). Additionally, detailed analyses of the various morphometric parameters (i.e., Sholl analysis and branching complexity) of individual apical and basal dendrites among TgCRND8 mice and wt controls showed no significant differences in dendrite length or number of intersections per radial distance from the soma (Fig. 3E,F).

TgCRND8 mice exhibit dendritic spine loss in CA1 neurons

Because impairments in cognitive function, but no changes in neuronal morphology, were observed in TgCRND8 mice, it is possible that static measurements of neuronal structure do not accurately reflect actual synaptic function. As such, we examined dendritic spine density type, as well as synapse density and type in the stratum lacunosum-moleculare of the CA1. Digitally reconstructed dendritic segments from TgCRND8 and wt mice are depicted in Figure. 4E-H. We found that, compared to controls, TgCRND8 mice have a significant decrease in apical spine density ($t_{(8)} = 2.451$; $p = 0.039$) with no differences in basal spine density ($t_{(8)} = 1.001$; $p = 0.346$; Fig. 4A,B). It has been demonstrated that different spine subtypes contain distinct properties and the proportion of spine subtypes is thought to reflect biological differences or changes in sensory input (Alvarez and Sabatini, 2007). Therefore, we systematically measured and categorized spines into three subtypes: stubby, thin, or mushroom. Our analysis revealed a significant interaction of genotype on spine type ($F_{(2,24)} = 4.13$, $p = 0.028$) with a decrease in thin spines and an increase in stubby spines on apical dendrites ($p < 0.05$) in transgenic mice compared to controls (Fig. 4C). Interestingly, while we did not observe changes in spine density in basal dendrites we did observe significant changes in basal dendritic spine types. As with apical dendrites, we observed an increase in stubby spines and a decrease in thin spines ($p < 0.001$; Fig. 4D). We next used EM to investigate spine ultrastructure further, and specifically to assess whether amounts of perforated versus non-perforated synapses are altered at this age in TgCRND8 mice. However, no difference was noted at the ultrastructural level between TgCRND8 mice and their wt littermates for levels of perforated or non-perforated synapses (Fig. 5).

Since spatial clustering of spines can affect neuronal function we assessed whether TgCRND8 mice exhibited specific clustering of spines using our established algorithm (Yadav et al., 2012) (Fig. 6A). The percentage of all spines that were spatially clustered did not differ between wt and transgenic mice (apical: $54.49 \pm 2.04\%$ vs. $56.87 \pm 2.07\%$, $p = 0.42$; basal: $55.36 \pm 1.61\%$ vs. $57.45 \pm 1.25\%$, $p = 0.32$). The distribution of clustered spines, as measured by the C-score, did not differ in wt versus TgCRND8 neurons (Fig. 6B,C; apical, $p = 0.84$; basal, $p = 0.46$). Moreover, in both groups, the frequency of clustered spines was no different from what was expected randomly (wt: apical $p = 0.36$, basal $p = 0.51$; TgCRND8: apical, $p = 0.41$, basal $p = 0.95$). In agreement with the spine type proportions reported above, there was a significant interaction of genotype on the clustering of apical spine type ($F_{(2,171)} = 12.73$, $p < 0.0001$) and basal spine type ($F_{(2,288)} = 16.43$, $p < 0.0001$) with a significant increase in the proportion of stubby spines and decrease in the proportion of thin spines which comprise clusters in transgenic versus control mice (Fig. 6D,E, apical: stubby, $p < 0.05$, thin, $p < 0.01$; basal: stubby, $p < 0.01$, thin, $p < 0.001$). Linear regression revealed that the type proportions measured across all spines on each branch accounted for 94% of the variation in the type proportions among clustered spines

(Fig. 6F, $C = 0.98 \cdot A + 0.75$, $r = 0.97$, $p < 0.001$). As such, it is unlikely that the detected spine clusters affected the distribution of spine types in the wt and TgCRND8 dendritic segments analyzed.

Altered levels of synaptic proteins in TgCRND8 mice

Next, we determined whether these changes in spine morphology were associated with alterations in synaptic protein expression. We analyzed levels of NMDA (GluN1; formerly NR1) and AMPA (GluA2; formerly GluR2) receptor subunits in the neocortex, hippocampus, and cerebellum of TgCRND8 vs wt mice in grossly dissected brain regions and compared between genotypes (Fig. 7). We noted a significant increase in GluN1 expression in 3month old TgCRND8 mice by comparison to wt littermates in all regions that were examined (Fig. 7A,B). Interestingly, we also noted a significant increase in expression of the AMPA receptor subunit GluA2 in the neocortex in TgCRND8 mice, while a significant decrease in GluA2 levels was observed in the hippocampus in TgCRND8 mice (Fig. 7A,C) GluA2 levels in the cerebellum were below the limit of reliable detection and were therefore not quantified.

Discussion

This study examined possible relationships between alterations in dendritic and spine morphology with changes in synaptic protein and behavioral impairment at early stages of AD pathology in an aggressive mouse model of brain amyloidosis. Prior studies using this mouse line have described the age of onset of plaque pathology and cognitive defects on hippocampus-dependent spatial memory tasks such as the MWM (Chishti et al., 2001; Hyde et al., 2005) or cued and contextual fear conditioning at later stages of pathology (Yang et al., 2011; Steele et al., 2013b). However, these studies did not include investigations of neuronal and spine morphology in the context of measurements of the molecular markers that are related to learning and memory-associated synaptic proteins.

Studies on the onset of cognitive deficits in AD mouse models, including the TgCRND8 model, are contentious in regards to onset of deficits. Here we report that by 3 months of age, TgCRND8 mice displayed defects in both contextual and cued fear memory (Fig. 1). Our data supports and validate further other studies indicating that TgCRND8 mice exhibit cognitive impairment on cued and contextual fear memory tasks as early as 4 months of age (Steele et al., 2013b) and at 6 months of age (Yang et al., 2011), or learning and spatial memory tasks by 3 months of age (Chishti et al., 2001; Hyde et al., 2005). In contrast, a recent report suggests that TgCRND8 mice do not display impairment of cued or contextual fear memory until 12 months of age (Hanna et al., 2012). Nonetheless, data from the current study, as well as others (Janus et al., 2000; Chishti et al., 2001; Hyde et al., 2005; Yang et al., 2011; Hanna et al., 2012; Steele et al., 2013b), suggest that TgCRND8 mice display behavioral impairment on tasks that require an intact function of both the amygdala and hippocampus as early as 3 months of age. What accounts for this impairment is still unresolved. However, the breakdown of hippocampal functions may be related to synaptic and neuronal toxicity that is associated with the accumulation of soluble and insoluble A β assemblies at this early stage of pathology.

Decrease in hippocampal neuron number may relate to onset of plaque pathology

It is known that with normal and pathological aging, changes in neuronal complexity and spine morphology and density correlate with cognitive deficits (reviewed in Dickstein et al., 2007 and Luebke et al., 2010, respectively). Based on prior indication of impairment on hippocampus-dependent tasks (e.g., MWM) in TgCRND8 mice at 3 months of age (Chishti et al., 2001; Hyde et al., 2005), and on our own data above, we chose to focus the current study on cellular and molecular alterations that underlie these defects in the hippocampus. We previously reported a significant decrease in hippocampal neurons in 6-month-old TgCRND8 mice by comparison to their wt littermates (Brautigam et al., 2012). Here we observed that by 3 months of age, TgCRND8 mice exhibit a significant decrease in number of hippocampal neurons (Fig. 2) compared to wt littermates. While we note that overexpression of the transgene product is associated with a reduced number of total hippocampal neurons at this age, we did not investigate the possible causes of this decrease. It should be noted that the Isotropic Fractionator is destructive and prevent analyses of neuronal numbers at a finer regional or laminar level. While our data show evidence of decrease in hippocampal neuronal numbers, we could not ascertain, without a formal stereologic assessment, whether this change was localized exclusively to the CA1 or reflected a more general phenomenon across the hippocampus.

We previously used the same technique to study a mouse model that develops only soluble assemblies of oA β , but never insoluble A β plaques (APP E693Q mouse line; Gandy et al., 2010), and observed no loss in hippocampal neurons (Brautigam et al., 2012). Therefore we postulate that cytotoxicity associated with insoluble A β (for review, see Lublin and Gandy, 2010) may result in the observed decrease in hippocampal neurons. It is however possible that this decrease may be due to altered stasis of adult hippocampal neurogenesis or to developmental defect (Mu and Gage, 2011). Also, because we have not studied neuronal morphology as a function of development we cannot exclude the possibility that the transgene products impair early neurogenesis at the time of initial brain development. Ongoing studies in our laboratory are aimed at determining whether the noted reduction in hippocampal neurons relates to perturbed development, altered adult hippocampal neurogenesis, or cell death due to the accumulation of insoluble A β assemblies.

In view of this neuronal loss we next examined if there were any morphological changes in CA1 pyramidal neurons. We found no significant differences in dendritic length and branching patterns between control and TgCRND8 neurons. Our results may differ from other studies due to different mouse models used, different brain regions examined, and differential inclusion or exclusion of neurons in relation to plaques. Dystrophic neurites, aberrant sprouting, varicosities, and abnormal dendritic curvature have been observed in pyramidal neuron closely opposed to or traversing A β plaques in the neocortex of many APP mutant mouse models (Knowles et al., 1999; Urbanc et al., 2002; Tsai et al., 2004; Spires et al., 2005). In contrast, another study found that frontal neurons from 12-month-old Tg2576 mice exhibited an increase in spatial extents, lengths, and volumes in both apical and basal dendritic arbors (Rocher et al., 2008). In this study, we examined neurons in the CA1 region of the hippocampus and did not distinguish between neurons next to or far from plaques.

Altered morphology of dendritic spines and levels of synaptic proteins in TgCRND8 mice at 3 months of age

Prior evidence suggests that subtle changes in synaptic morphology relate to early cognitive defect in the Tg2576 mouse model of AD (Jacobsen et al., 2006). Here, we used multiple methods of measuring synaptic morphology and biochemistry to evaluate changes that may be associated with early stage AD-related neuropathology in the CA1 region of the hippocampus in the TgCRND8 mouse. Our results on neuronal morphology suggest that there is no difference in the dendritic integration of synaptic inputs in CA1 neurons of 3-month-old control and TgCRND8 mice. Rather, any differences in signal integration are likely due to differences in spine density and morphology (Araya et al., 2006a; Araya et al., 2006b; Gullledge et al., 2012). It has been demonstrated that different spine subtypes contain distinct properties and the proportion of spine subtypes is thought to reflect biological differences or changes in sensory input (Radley et al., 2006; Alvarez and Sabatini, 2007; Radley et al., 2008; Tampellini and Gouras, 2010; Bloss et al., 2011; Hara et al., 2011). Moreover, recent studies have shown that clustered groups of dendritic spines can increase a neuron's ability to integrate and differentiate its inputs (see Govindarajan et al., 2006, and Larkum and Nevian, 2008, for review). In particular, nearby spines can support nonlinear summation of inputs (Larkum and Nevian, 2008; Larkum et al., 2009), exhibit coregulation during the induction of LTP (Harvey and Svoboda, 2007; De Roo et al., 2008; Harvey et al., 2008; Govindarajan et al., 2011), and process functionally similar inputs (Takahashi et al., 2012). We systematically measured and categorized spines into three subtypes: stubby, thin, or mushroom and assessed the spatial clustering of spines which may correspond to functional groups. Our analysis revealed a significant decrease in thin spines and an increase in stubby spines in apical dendrites in transgenic mice compared to controls (Fig. 4C), with analogous changes in the proportion of thin and stubby spines, which comprise clusters in transgenic versus control mice (both apical and basal; Fig. 5D,E).

Because spine morphology (i.e., mushroom vs thin or stubby spines) and molecular architecture of synapses (e.g., receptor levels and localization in dendritic spines) typically determines the strength and stability of those connections (Radley et al., 2006; Radley et al., 2008; Bloss et al., 2011), we propose that the noted shift in structure from thin to stubby spines and altered expression of GluN1 (increased) and GluA2 (decreased) in the hippocampus may reflect subtle, but lasting, changes in ability of these neurons to form connections that will mature from “new” to “stable” synapses in the presence of high levels of A β . By extension, we propose that these subtle alterations may represent one underlying mechanism of A β -related toxicity, although future studies will be required to determine the size and species of A β structures, as well as changes in subcellular localization of specific NMDA and AMPA receptor subunits (i.e., GluN1, GluN2B, GluA1/2/3) at the synapse, and their effects on cellular mechanisms such as long-term potentiation (LTP) or depression, and cognitive phenotypes over time in the TgCRND8 and other AD mouse models. In fact, the reduction in hippocampal GluA2 levels that we observe is interesting in light of prior reports demonstrating a decrease in AMPA receptors in AD (Thorns et al., 1997; Aronica et al., 1998; Bloss et al., 2011) and impairment in LTP (Jacobsen et al., 2006). There is still much debate in regards to NMDA receptor changes in AD. Whilst some indicate no changes in NMDA expression and thus excitatory postsynaptic currents (EPSCs) (Ting et al., 2007)

others note reductions in NMDA expression and NMDA-mediated EPSCs (Kanju et al., 2007; Priller et al., 2009).

In summary, the present study provides a multifaceted framework for future studies aimed at further elucidating the relationship between early AD-like A β neuropathology, behavioral defect, and changes in neuronal and synaptic morphology. We provide evidence of behavioral deficits among TgCRND8 mice, accompanied by a reduced number of neurons in the hippocampus by comparison to wt littermates, at an early stage of AD-like neuropathology. Through detailed study of neuronal and synaptic morphology, ultrastructure, synaptic clustering analysis, and biochemical analysis of synaptic glutamate receptors, we noted a shift from a typically “stable” to “labile” phenotype of both apical and basal dendritic spines in the hippocampus. These data suggest that even at this early stage of disease, there is a perturbation of neuronal maintenance and homeostasis that may result from aberrant neurogenesis and/or cellular death. Based on these findings, we propose that early and long-lasting changes in neuronal connectivity in the hippocampus may underlie the appearance of clinically obvious symptomatology. If so, then the 3-month-old TgCRND8 mouse may offer a useful *in vivo* system for the discovery of novel targets for development of AD therapeutics.

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Abbreviation

A β Amyloid- β protein

AD	Alzheimer's disease
A	Amyloid precursor protein
CB	Cerebellum
DAPI	4',6-diamidino-2 phenylindole
EM	Electron microscopy
HPC	Hippocampus
MWM	Morris water maze
NC	Neocortex
PBS	Phosphate-buffered saline
PSD	Postsynaptic density
oAβ	Oligomeric assemblies of A β
wt	Wild-type

Figure 1.

TgCRND8 mice develop plaque pathology and impaired fear memory by 3 months of age.

(A) Immunofluorescence staining with Pan1G6 anti-Aβ₄₂-C-terminus antibody (green) reveals Aβ₄₂-laden plaque pathology in the neocortex and hippocampus of a 3-month-old TgCRND8 mouse. DAPI (blue) was used as counterstain. Scale bar = 1 mm. (B)

Hippocampi were dissected from 3-month-old TgCRND8 (n = 5) or wt littermates (n = 5) mice and analyzed for levels of soluble and insoluble APP metabolites by Western blot. Western blots are from representative littermates indicating high levels of transgene

expression in the TgCRND8 mice. (C) 3-month-old male TgCRND8 (n = 18) and wt littermates (n = 19) were tested for cued and contextual fear memory, and TgCRND8 mice displayed significantly impaired cued and contextual fear memory by comparison to wt littermates. Graphs represent means ± SEM; *, p < 0.001.

Figure 2.

TgCRND8 mice exhibit significant neuronal loss in the hippocampus as early as 3 months of age. There was a significant decrease in number of NeuN-immunoreactive neurons in the hippocampus (B) of TgCRND8 mice compared to wt, however, not in the cerebellum (A) or neocortex (C). No changes in total cell numbers (D-F) were observed in the brain of TgCRND8 compared to wt mice (wt, n = 8; TgCRND8, n = 7 animals per group, $p < 0.05$). Data presented represents group mean $\pm$ SEM.

Figure 3.

No alterations in neuronal morphology with amyloid pathology. Representative examples of 3D reconstructed CA1 neurons and corresponding apical dendrite dendrograms from wt (A) and TgCRND8 (B) mice. No changes were observed throughout apical and basal trees in dendritic length (C,D) and in dendritic tree complexity (E-H) between wt (n = 5) and TgCRND8 (n = 5) mice. Data presented represents group mean $\pm$ SEM. Scale bar = 50 μ m.

Figure 4.

Changes in spine plasticity in AD model mice. Amyloid pathology was associated with significant reductions in spine density throughout the apical (A) but not basal (B) trees in TgCRND8 mice ($n = 5$) compared to wt ($n = 5$). Changes in spine types were also observed with an increase in apical stubby spines (C) and an increase in basal stubby and decrease in basal thin spines (D) in TgCRND8 mice compared to wt. (E-H) representative dendritic segments from wt (E apical; F basal) and transgenic (G apical; H basal) CA1 neurons. Scale bar = 5 μm . Bar graphs represent group mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.001$ compared to controls.

Figure 5.

Electron microscopy studies revealed no significant changes in the perforated versus non-perforated synapses. (A) Representative EM images from wt (left) and TgCRND8 (right) mice (• denote single synapses; ★ denotes perforated synapses). Perforated synapses were classified by the presence of a discontinuity (or more than one) within the PSD. Scale bar = 500 nm. No significant changes in perforated density (B) or non-perforated density (C) were observed between wt (n = 5) and TgCRND8 (n = 5) mice. Data represent group mean $\pm$ SEM.

Figure 6.

Spatial clustering of dendritic spines is similar in control and AD model mice. (A) Spatial clusters of spines identified along a CA1 dendrite branch segment from a wt mouse, overlaid on the xy- and xz-projections of the deconvolved image. Colors denote spines within the same cluster; unclustered spines are shown in white. Scale bar = 2 μ m. (B,C) The cumulative distribution of C-scores in control and TgCRND8 mice in apical (B) and basal (C) branches, in solid black and dashed lines respectively. Thin lines indicate the cumulative distribution function of C-scores following a normal distribution with the same mean and standard deviation as the empirical data. (D,E) The proportion of stubby, thin, and mushroom spines within spine clusters in wt and TgCRND8 branches of apical (D) and basal (E) arboris. Bar graphs represent group mean $\pm$ SEM. *, $p < 0.05$ and **, $p < 0.01$ compared to controls. (F) Percentage of each spine type among clustered spines versus all spines, for all apical branches analyzed. Stubby, thin, and mushroom percentages are shown as squares, triangles, and circles respectively. Data from the control group is shown as filled black symbols; TgCRND8 data shown as open symbols. Regression line was computed from both apical and basal data together.

Figure 7.

TgCRND8 mice exhibit region-specific alterations in synaptic proteins compared to wild-type littermates. (A) Representative Western blots and analysis of band integrated density (B,C) of brain lysates from the neocortex, hippocampus, and cerebellum revealed an increase in GluN1 NMDA receptor protein levels in all brain regions, an increase in GluA2 in the cortex, and a decrease in GluA2 in the hippocampus in TgCRND8 mice versus wt mice. NC = Neocortex, HPC = Hippocampus, CB = Cerebellum; GluA2 levels were below the limit of reliable detection in the cerebellum and were therefore not quantified. Histograms represent means $\pm$ SEM. *, $p < 0.001$

Table 1
Antibodies used in this study

Antigen	Immunogen	Host species	Source/cat. no	Dilution
pAb 369	pan-species C-terminus of β APP ⁶⁴⁵⁻⁶⁹⁴ (VAPAVPAVSLVPPAFPVS MPVPPPGFNPIPPPPFLRA SFNPSQPPPGFMP)	Rabbit (polyclonal)	(Buxbaum et al., 1990; Gandy et al., 1998)	1:4,000
pAb 1G6	anti-A β ₄₂ -C-terminus	(monoclonal)	(Buxbaum et al., 1990; Steele et al., 2012)	1:500
6E10	Human-specific aa 1-16 of A β domain. The epitope lies within aa 3-8 of A β (EFRHDS)	Mouse (monoclonal)	Covance; SIG-39320	1:1,000
anti- β -Actin	Slightly modified β -cytoplasmic actin N-terminal peptide conjugated to KLH (DDIAALVIDNGSGK)	Mouse (monoclonal)	Sigma; clone AC-15, A1978	1:20,000
Anti-GluN1	synthetic peptide corresponding to the C-terminus of rat GluN1 (K-QYHPTDITGPLNLS DPSVS TVV)	Rabbit (polyclonal)	Millipore; 06-314	1:2,000
Anti-GluA2	Synthetic peptide of rat GluA2 conjugated to BSA through a cysteine added to the C-terminus of the peptide	Rabbit (polyclonal)	Millipore; AB1768; Lot# LV1478919	1:1,000
anti-NeuN	Purified cell nuclei from mouse brain	Mouse (monoclonal)	Millipore; clone A60, MAB377	1:200
IgG-Alexa-Fluor 594		Goat	Millipore	1:200
IgG-Alexa-Fluor 488		Goat	Millipore	1:200