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AMYLOID BETA OLIGOMERS IMPAIR FEAR CONDITIONED MEMORY IN A CALCINEURIN-DEPENDENT FASHION IN MICE

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Abstract

Soluble oligomeric aggregates of the amyloid beta (A β) peptide are believed to be the most neurotoxic A β species affecting the brain in Alzheimer Disease (AD), a terminal neurodegenerative disorder involving severe cognitive decline underlined by initial synaptic dysfunction and later extensive neuronal death in the CNS. Recent evidence indicates that A β oligomers are recruited at the synapse, oppose expression of long term potentiation (LTP), perturb intracellular calcium balance, disrupt dendritic spines and induce memory deficits. However, the molecular mechanisms behind these outcomes are only partially understood; achieving such insight is necessary for the comprehension of A β -mediated neuronal dysfunction. We have investigated the role of the phosphatase calcineurin (CaN) in the pathological processes of AD. CaN is especially abundant in the CNS where it is involved in synaptic activity, LTP and memory function. Here, we describe how oligomeric A β treatment causes memory deficits and depresses LTP expression in a CaN-dependent fashion. Mice given a single intracerebroventricular injection of A β oligomers exhibited increased CaN activity and decreased pCREB, a transcription factor involved in proper synaptic function, accompanied by decreased memory in a fear conditioning task. These effects were reversed by treatment with the CaN inhibitor FK506. We further found that expression of hippocampal LTP in acutely cultured rodent brain slices was opposed by A β oligomers and that this effect was also reversed by FK506. Collectively, these results indicate that CaN activation may play a central role in mediating synaptic and memory disrupting effect induced by acute oligomeric A β treatment in mice.

Keywords

Alzheimer Disease; PP-2B; LTP; pCREB

INTRODUCTION

Alzheimer Disease (AD) is a neurodegenerative disorder involving severe cognitive decline that is characterized by initial synaptic dysfunction and later extensive neuronal death in the CNS (Selkoe 2002). A hallmark of AD is the presence of extracellular amyloid plaques, formed by aggregated amyloid beta (A β), a 40- or 42-aminoacid long peptide resulting from cleavage of the larger amyloid precursor protein (APP) (Golde 2005; Racchi and Govoni

2003). A β is prone to misfolding and aggregating into soluble oligomers, which eventually assemble into the large amyloid fibrils found in AD senile plaques (Adlard et al. 2009). Evidence suggests that soluble A β oligomers are the most toxic A β species in the AD brain (Glabe 2006; Kaye et al. 2003; Walsh and Selkoe 2004; Walsh and Selkoe 2007). For example, in AD patients the levels of soluble A β correlate with disease severity, while plaques burden does not. (Lue et al. 1999; McLean et al. 1999; Naslund et al. 2000). Furthermore, APP transgenic mice develop memory deficits when oligomeric A β is abundant and months before plaques are detected (Gordon et al. 2002; Kawarabayashi et al. 2001; Reese et al. 2008).

This correlative evidence implies that oligomeric A β is the form which negatively impacts synaptic integrity and leads to functional deficits of memory. Many lines of *in vitro* and *in vivo* research have converged to support this hypothesis. A β oligomers, but not monomers or fibrils, increase intracellular Ca²⁺ levels and cell death in human S5Y5 neuroblastoma cells (Demuro et al. 2005; Reese et al. 2008). Oligomeric A β isolated from APP transgenic mice (Lesne et al. 2006), human AD brains (Shankar et al. 2008) and from the culture medium of chinese hamster ovary (CHO) cells overexpressing human APP (Cleary et al. 2005) are known to reversibly disrupt memory when injected intracerebroventricularly (icv) in rodents. Indeed, A β oligomers inhibit the induction and expression of long term potentiation (LTP) in the hippocampus, both *ex vivo* (Shankar et al. 2008; Wang et al. 2002) and *in vivo* (Townsend et al. 2006). These effects are not surprising given the propensity of oligomers to accumulate at the synapse (Koffie et al. 2009; Lacor et al. 2004) where they promote alterations of intracellular Ca²⁺ which are associated altered synaptic architecture and dysregulated intracellular signaling including activation of the calcium-dependent phosphatase calcineurin (CaN) activation (Kuchibhotla et al. 2008; Reese et al. 2008; Shankar et al. 2007).

Collectively, this evidence indicates a central role for oligomers in A β -induced cognitive decline; however, the exact molecular pathways involved remain elusive. We have previously shown that APP transgenic mice (Tg2576) display increased CaN activity in the CNS at 5 months, an age that precedes plaque formation but coincident with the onset of cognitive deficits as measured by the fear conditioning memory task and in the novel object recognition memory test (Dineley et al. 2007; Taglialetela et al. 2009). In those studies, we showed that acute treatment with the CaN inhibitor FK506 reversed these cognitive impairments in Tg2576 mice. We further found that A β oligomers, but not monomers or fibrils, increase CaN activity in human SY5Y neuroblastoma cells and cultured rodent brain slices. CaN activation subsequently leads to decreased levels of phosphorylated and transcriptionally competent CREB (pCREB) (Reese et al. 2008), a transcription factor downstream of CaN essential to synaptic plasticity and memory (Bito et al. 1996; Pittenger and Kandel 1998; Waltereit and Weller 2003). In all cases, treatment with FK506 rescued decreased pCREB levels (Reese et al. 2008).

As such, CaN over-activation appears to be an important component of cognitive dysfunction induced by oligomeric A β in AD. To directly address if the oligomeric form of A β is an important trigger for CaN-dependent cognitive dysfunction, we determined the extent of CaN over-activity and fear conditioning memory deficits induced by an acute icv injection of A β oligomers, in wild type mice, and the effect thereupon of pharmacological inhibition of CaN.

METHODS

Materials

Antibodies against CREB, p(Ser133)CREB, mTOR, p(Ser2448)mTOR and p(Thr389)p70-S6K were from Cell Signaling Technology, Danvers, MA, and the anti-A β antibody 6E10 was from Convence, France. FK506 and rapamycin were purchased from LC Laboratories, Woburn, MA. Human Amyloid beta 1–42 was purchased from the Dept. of Biophysics and Biochemistry, Harvard University, New Haven, CT. All other reagents were molecular biology purity grade and were purchased from Sigma Chemicals unless otherwise noted.

Preparation of A β

To prepare oligomeric A β , lyophilized A β aliquots (0.3 mg) were dissolved in 0.2 ml of 1,1,1,3,3,3-Hexafluoro-2-propanol (HFP) and then added to 0.7 ml of H₂O. Samples were loosely capped and stirred on a magnetic stirrer under a fume hood for 48 hr and then used within 36 hr. Quality of A β preparations so obtained were routinely checked by dot-blot and western blot employing both A-11 and 6E10 antibodies. Occasionally, the structural appearance of oligomeric A β preparations was checked by electron microscopy to visually exclude the presence of contaminant fibrils.

The final concentration of the oligomeric A β preparation was nominally calculated based on the concentration of the starting A β monomer.

Animals

For behavioral studies, male C57BL/6 mice at 5 months of age were used, whereas for brain slice preparations, male Sprague Dawley rats (150–250 g) were used. Animals were maintained at the UTMB vivarium under USDA standards (12 hr light/dark cycle, food and water *ad libitum*).

At the end of each experiment, animals were sacrificed by exposure to halothane vapors followed by decapitation. Brains were rapidly removed, dissected into individual areas including hippocampus, cerebellum, midbrain, hypothalamus, striatum, frontal cortex and occipital cortex and stored at –80°C until further biochemical analyses were performed.

Intracerebroventricular (icv) injection

Following an IACUC-approved protocol, injections icv were performed via a free-hand method modified from Clark et al. (Clark et al. 1968). Briefly, mice were deeply anesthetized with a ketamine/xylazine mixture (65 and 7.5 mg/kg ip, respectively), the scalp was shaved and an incision was made through the midline to expose the top of the skull. A 28 gauge needle held with a haemostatic forceps to leave 3 mm of the needle tip exposed was lowered 1 mm posterior and 1 mm lateral of the bregma. The needle was connected through a 0.28 mm polyethylene tubing to a 25 μ l syringe driven by an electronic programmable micro-infuser (Harvard Apparatus, Holliston, MA) which was used to deliver 3 μ l/mouse at a rate of 6 μ l/min. After injection, the needle was left in place for 1 min and the surgical wound stitched before placing the mouse to recover while lying on a heated pad under a warm light. Reliability and consistency of intraventricular injections was routinely tested during actual experiments by injecting India ink in parallel blank animals and macroscopically observing proper coloration of the ventricles (Supplementary Fig. 1).

Fear Conditioning

Six hrs before the training session of the behavioral test, half of the mice were treated with FK506 (10 mg/kg ip). In separate experiments, mice were treated with rapamycin (5 mg/kg

ip) instead. Age and sex matched animals were injected with a comparable volume of vehicle (1% DMSO in 0.9% saline). There were 10 animals per treatment group. The standard protocol consisted of a training phase when the mice were placed in a particular environment (a training chamber with particular lighting, geometry, odor that constitutes the context conditioned stimulus, CS) and allowed to explore for 3 min. An auditory CS (80dB white noise) was then presented for 30 sec and one footshock (0.8 mA, 2 sec duration; the unconditioned stimulus, US) delivered during the last 2 sec of the auditory CS. A second presentation of the auditory CS and the US was delivered at the 5 min mark and the animals then left in the cage for another 2 min. Twenty-four hours later, the mice were returned to the same training chamber and the context test for fear learning performed. The amount of freezing the mice exhibited during 5 min in the training chamber was measured. One hour later the cued test was performed in a completely novel context. The animals were placed in the testing chamber and freezing was measured for 3 min before the auditory CS was re-presented and freezing quantified over the next 3 min.

Phosphatase activity assay

The activity of CaN (PP-2B) and combined activity of PP-1/PP2A were assayed in protein extracts using a commercially available colorimetric kit (EMD Biosciences, San Diego, CA) employing R2 phosphopeptide as substrate according to the manufacturer's instructions.

Western blot

Tissues were homogenized in SDS lysis buffer containing 5mM EDTA, 50mM Tris, 2% SDS, 1mM DTT, 1mM PMSF and 1% protease cocktail inhibitors (Sigma, St. Louis, MO), centrifuged at 20,000g for 5 minutes and the supernatants collected. Protein samples containing 30 µg of proteins, as determined by the BCA assay (Pierce, Rockford, IL), were subjected to SDS-PAGE using 12% gels, followed by electrophoretic transfer to nitrocellulose backed membranes (Biorad, Hercules, CA). Membranes were then incubated with appropriate dilutions (usually 1:1,000 v:v) of the primary antibody and this initial incubation was then followed by a horseradish peroxidase (HRP) conjugated secondary antibody (Biorad, Hercules, CA) against rabbit IgG (for polyclonal primaries) or mouse IgG (for monoclonal primaries). Detection was achieved using ECL (Pierce, Rockford, IL) and quantification of band intensity was performed via a densitometry software (AlphaEase) applied to digitally captured autoradiography images of gels.

Immunohistochemistry

Whole mouse brains were rapidly frozen in liquid nitrogen and stored at -80°C . Prior to sectioning, brains were equilibrated to -25°C before embedding in O.C.T. compound (Tissue-Tek). Ten µm sections were cut and affixed to Superfrost Plus slides (Fisher Scientific), for storage at -20°C . After briefly equilibrating to room temperature, sections were rinsed in 0.1M PBS and then fixed in ice-cold 4% paraformaldehyde. After two brief washes in 0.1M PBS, the sections were blocked and permeabilized for 1 hour in 0.1M PBS containing 10% goat serum, 0.03% Triton-X, and 0.1% phosphatase inhibitor (Pierce). Incubation with primary antibodies (mouse Mab377 for NeuN, 1:1000 v:v dilution, and rabbit 06-519 for pCREB, 1:100 v:v dilution, both from Millipore, Billerica, MA) in 0.1M PBS containing 10% serum was overnight at room temperature. Following two washes, the slides were incubated with Alexa Fluor 488 and 594 secondaries (Invitrogen) in 0.1M PBS containing 10% serum. Slides were again rinsed twice in PBS and twice in distilled water before Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) was applied, and coverslips were mounted. Nail polish was applied to seal the edges and maintain signal.

Microscopy

Data was acquired with a laser scanning system (Bio-Rad Radiance 2100, K-2 system). Images were collected with krypton lasers of 488 nm excitation and 568 nm excitation. For DAPI a blue diode was used. Data was collected by sequential scans to avoid bleeding through between the three channels. Single images were collected at scan speeds of 25 lines per second at 1024×1024 pixel resolution.. The acquisition parameters were kept constant for all scans in the same experiment.

Brain slice preparation

According to IACUC-approved protocols, rats were decapitated and brains quickly removed and blocked in cold (4°C) artificial CSF (ACSF) prepared as follows: 117 mM NaCl, 4.7 mM KCl, 1.2 mM Na₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 25 mM NaHCO₃, 11 mM glucose, oxygenated and equilibrated to pH 7.4 with 95% O₂/5% CO₂. Five-hundred μm thick coronal brain slices comprising the hippocampus were prepared using a Vibroslice (Camden Instruments, London, UK) and equilibrated in ACSF at room temperature for at least 30 min prior to adding test compounds. Treatment with Aβ started 1 hr prior to recording, at the beginning of which Aβ was removed. FK506 or rapamycin treatment started 15 min prior to recording and was continued throughout the recording period. At the end of the experiments (electrophysiological recording), slices were snap frozen in liquid nitrogen and stored at -80°C until further analyses were performed.

Patch-clamp recordings

A single brain slice was transferred to the recording chamber and submerged in ACSF (31°C), which superfused the slice at ~2 ml/min. Only one neuron was recorded in each slice, and a fresh slice was used for each new experimental protocol. Numbers in the text refer to the number of neurons tested for each parameter. Whole-cell recordings using the blind-patch technique or DIC-enhanced infrared (IR)-videomicroscopy were done as described previously (Fu and Neugebauer 2008; Neugebauer et al. 2003). Recording pipettes (3–5 MΩ tip resistance) made from borosilicate glass (1.5 mm o.d., 1.12 mm i.d.; Drummond) were filled with intracellular solution containing (in mM): 122 K-gluconate, 5 NaCl, 0.3 CaCl₂, 2 MgCl₂, 1 EGTA, 10 HEPES, 5 Na₂-ATP, and 0.4 Na₃-GTP; pH was adjusted to 7.2–7.3 with KOH and osmolarity to 280 mOsm/kg with sucrose. Recording electrodes were positioned in hippocampal CA1 region under visual control. The boundaries of the hippocampal areas are easily discerned under light microscopy. After tight (>2GΩ) seals were formed and the whole-cell configuration was obtained, neurons were included in the sample if their resting membrane potential was more negative than -50 mV and action potentials overshooting 0 mV were evoked by depolarizing current injections. Data acquisition and analysis of voltage and current signals was done using a dual 4-pole Bessel filter (Warner Instr.), low-noise Digidata 1322 interface (Axon Instr.), Axoclamp-2B amplifier (Axon Instr.), Pentium PC, and pClamp9 software (Axon Instr.). Headstage voltage was monitored continuously on an oscilloscope to ensure precise performance of the amplifier. Neurons were voltage-clamped at -60 mV. Seal and series resistances were checked throughout the experiment (using pClamp9 membrane test function) to ensure high-quality recordings.

Synaptic transmission and LTP induction

Synaptic transmission was measured at the Schaffer collateral/commissural pathway that provides glutamatergic input to CA1. Using a concentric bipolar stimulating electrode (Kopf Instruments) of 22 kΩ resistance, excitatory postsynaptic currents (EPSCs) were evoked in CA1 neurons by focal electrical stimulation (Grass S88 stimulator, Grass Instr.) of the Schaffer collateral/commissural pathway. Test EPSCs were evoked by electrical stimuli

(200 μ s square-wave pulses) delivered at a frequency of 0.033 Hz. Stimulus intensity was set to evoke a half-maximal response (50 % of EPSC amplitude). LTP was induced using high-frequency stimulation (HFS) consisting of 3 trains at 100 Hz (1 s duration each; intertrain-interval of 20 s). During HFS stimulus, intensity was raised to evoke an EPSC that was 75% of the maximum amplitude.

Statistical analysis

Statistical analysis of behavior data was accomplished with either one-way or two-way ANOVA (Prism, GraphPad software), followed by Bonferroni post hoc multiple comparisons test. A value of $p < 0.05$ was considered significant. For paired analyses, Students' t-test was performed. Where appropriate (unequal variance amongst groups), Bonferroni correction for four-way comparisons was done by reducing the p value for significance to 0.0125.

RESULTS

To determine the effect of A β oligomers on memory as a function of CaN activation, we icv-injected wild type mice with A β oligomers or vehicle and subjected them to the FC memory test. A schematic of the experimental design is shown in Fig. 1A. A β oligomers were freshly prepared from synthetic human A β ₁₋₄₂ as previously described (Kayed et al. 2003; Reese et al. 2008). This preparation typically yields a mixture of A β oligomer species, including dimers, trimers and dodecamers.

At hour eighteen following icv injection, half of the mice received an intraperitoneal (ip) injection of either the CaN inhibitor FK506 or vehicle; FC training occurred at hour twenty-four, with FC testing (contextual and cued) and sacrifice at hour forty-eight. Brains were dissected and tissue was used for biochemical analyses immediately thereafter. Compared to mice that received vehicle icv, those given A β oligomers icv and vehicle ip showed impaired FC memory. This deficit was more pronounced at 120 seconds and later time points of the test duration (Fig. 1B, upper panel). On the other hand, animals that received A β oligomers icv and FK506 ip 6 hr prior to FC testing performed equally well as control mice. Cumulative analysis of FC data (Fig. 1B, lower panel) confirmed statistically that mice receiving A β oligomers icv and vehicle ip showed FC memory deficits as compared to control mice or A β oligomer-injected mice that were further treated with FK506 ip prior to FC training. On the other hand, the same mice injected with A β oligomers icv and vehicle ip showed no impairment in the cued FC memory test as compared to control or A β oligomer/FK506-injected animals (Fig. 1C).

To confirm specificity of FK506 treatment, a separate group of mice was treated with A β oligomers icv followed by ip injection with rapamycin in a parallel experimental design as schematically depicted in Fig. 1A. Rapamycin is a macrolide immunosuppressant similar to FK506 in that it inhibits interleukin-2 signaling, without the additional inhibition of CaN (Abraham and Wiederrecht 1996); thus, any effect elicited by FK506 but not rapamycin is highly likely to be exerted through inhibition of CaN. We found that contextual and cued FC memory in A β oligomer-treated mice that received rapamycin did not differ significantly from animals who received A β oligomers icv and vehicle ip prior to FC training (Supplementary Data Fig. 2A and 2B). These results cannot be attributed to the ineffectiveness of rapamycin, as a Western blot in the hippocampus of these mice reveals decreased phosphorylation of the rapamycin target mTOR (Abraham and Wiederrecht 1996) and its downstream target pS6K (Kinnard et al. 2005) as compared to FK506-injected mice (Supplementary Data Fig. 3A and 3B).

When phosphatase activity was measured in the hippocampus of mice collected at the end of the FC experiment described in Fig. 1A, we found that animals injected with A β oligomers had increased CaN activity as compared to animals that received vehicle icv and that such an increase was reversed by FK506 treatment (Fig. 1D). Neither A β oligomers nor FK506 had any significant effect on the combined activity of hippocampal PP1 and PP2A, two phosphatases that at variance with CaN are calmodulin-independent (Mansuy and Shenolikar 2006). Levels of CaN expression as determined by Western blot were unchanged in the hippocampus of these mice (not shown).

A separate group of mice were given vehicle or oligomeric A β icv, and 18 hr later either vehicle or FK506 was administered ip. At 24 hr the mice were sacrificed and hippocampi (for Western blot analysis) or whole brains (for IHC studies) collected (Fig. 2). The timing of this experiment was chosen to determine the influence of A β oligomer injection on the levels of pCREB, a transcription factor modulated by CaN and crucial to synaptic plasticity and memory function (Bito et al. 1996; Pittenger and Kandel 1998; Waltereit and Weller 2003), at a time when FC memory is formed (i.e., at time of FC memory training). We found that A β oligomer-treated mice had lower pCREB levels in the hippocampus as compared to vehicle-injected mice (Fig. 2A); we further observed that FK506 reversed this phenomenon, indicating CaN involvement in A β oligomer-induced reduction of hippocampal pCREB. No changes in either pCREB or CaN protein levels were noted in other CNS areas examined, including midbrain, cortex and cerebellum (not shown). IHC staining for NeuN and pCREB (Fig. 2B) revealed that reduction of pCREB was most pronounced in neurons throughout the hippocampus and particularly the CA3 region and the dentate gyrus. This phenomenon was reversed by treatment with FK506, thus confirming Western blot data.

Increase of CaN activity and reduction of pCREB in the hippocampus of memory impaired, A β oligomer-treated mice would suggest altered synaptic plasticity, where CaN hyperactivity may play a determinant role in mediating memory deficits. In order to investigate the role of CaN in synaptic dysfunction induced by oligomeric species of A β , we measured expression of hippocampal LTP in brain slices treated with A β oligomers followed by treatment with FK506 (Figs. 3A and 3B). Recording conditions (seal resistance), membrane properties (resting membrane potential, input resistance), and baseline synaptic transmission (EPSC thresholds, maximum amplitude, and 10–90% rise time) were not significantly different between neurons in control slices and in slices subjected to the different treatments. Confirming previous literature evidence (Selkoe 2008; Shankar et al. 2008; Townsend et al. 2006; Walsh et al. 2002), expression of LTP was abolished in brain slices treated with A β oligomers 1 hr prior to recording as compared to control slices. Treatment with FK506 effectively reversed the abolishment of LTP expression induced by oligomeric A β . Rapamycin was not effective in negating the effect of oligomeric A β nor did it interfere with FK506 rescue, further indicating separate signaling pathways affected by the two drugs. Indeed, when we measured phosphatase activity in the very same slices used for LTP recording we found that A β oligomers significantly increased CaN activity without affecting the combined activity of PP1 and PP2A; this effect was abolished by FK506 but not rapamycin. Again, rapamycin did not affect normalization of CaN activity when administered in conjunction with FK506. Also in this experiment, we confirmed pharmacological efficacy of rapamycin by measuring pS6K phosphorylation in brain slices treated with rapamycin as compared to control slices or slices treated with FK506 (Supplementary Data Fig. 3C).

DISCUSSION

Taken together, the results from this study indicate that A β oligomers delivered to the CNS of wild type mice increase CaN activity and produce CaN-dependent memory deficits in the

FC memory task, likely through a mechanism involving reduced neuronal pCREB and decreased LTP in the hippocampus. Our data further indicate that pharmacological inhibition of CaN may effectively oppose these neurobehavioral dysfunctions elicited by oligomeric A β .

Administration of A β oligomers significantly impaired FC memory scores in mice. However, animals that received A β learned the task (associating the auditory stimulus with the upcoming foot-shock) during the training session as readily as vehicle-injected mice (not shown). Therefore, we conclude that A β treatment precludes formation of new memories and their recollection, rather than disrupting the ability to learn. Indeed, we found that mice injected with A β oligomers had increased hippocampal CaN and decreased pCREB, two elements that reportedly play essential and opposing roles (negative and positive, respectively) in the formation of new memory via synaptic plasticity (Impey et al. 1998; Mansuy et al. 1998). Consistent with this, we previously found that A β oligomers, but not monomers of fibrils, decrease pCREB in acutely cultured rodent brain slices (Reese et al. 2008). In the present study, we show that in the same *ex vivo* model A β oligomers increase CaN activity and oppose expression of LTP, the widely recognized cellular basis of memory acquisition (Pittenger and Kandel 1998).

Our current results place CaN centrally within the mechanisms mediating oligomeric A β -induced memory dysfunction. Systemic administration of the CaN inhibitor FK506 reduces hippocampal CaN hyper-activation and rescues pCREB reduction induced by A β oligomers while restoring cognitive function as measured by the FC memory task. In addition, our results show that inhibition of CaN by FK506 also rescues the deficit of hippocampal LTP expression acutely induced by A β oligomers in organotypic rodent brain slices. These observations thus indicate that an increase in CaN activity is a necessary step mediating the synaptic and behavioral effects of oligomeric A β . Importantly, in both our *in vivo* and *ex-vivo* experiments, FK506 was delivered following the treatment with A β oligomers (18 hr and 45 min delay for mouse and brain slice treatments, respectively) and shortly before FC training (6 hr) or LTP recording (15 min). This indicates that i): the observed A β oligomer-induced changes in synaptic and memory function were not due to cell damage (e.g., neuron death) or other permanent modifications, and ii): acute inhibition of CaN reversed (rather than prevented) the behavioral toxicity of A β oligomers. This is consistent with our previous results showing that a single injection of FK506 completely restored decreased hippocampal pCREB levels and reversed FC memory deficits in Tg2576 APP transgenic mice with increased CaN (Dineley et al. 2007). In a subsequent study, we observed that decreased pCREB in the hippocampus of 5 month old Tg2576 mice was associated with the presence of A β oligomers (Reese et al. 2008). We further found that acutely treating these mice with FK506 rescued hippocampal pCREB without affecting A β oligomer levels, implying that CaN hyperactivation lies downstream of A β oligomer action. These previous studies in Tg2576 mice indirectly indicated that CaN may be central to mediating reversible effects of A β oligomers in the CNS leading to memory dysfunction. Our current results in wild-type mice provide direct evidence supporting this hypothesis, demonstrating that decreased hippocampal pCREB and impaired memory in the FC task induced in mice by exogenously administered A β oligomers may be reversed by acute inhibition of over-activated CaN. However, while our previous and current results indicate that de-phosphorylation of pCREB always accompanies A β -induced CaN up-regulation in *in vitro*, *ex vivo* and *in vivo* experimental models, thus suggesting a central role for a CaN/pCREB signaling system, it is important to notice that alterations of CaN activity perturb other significant cellular and signaling events pertinent to CNS function. These include regulation of pro-inflammatory genes in activated astrocytes (Norris et al. 2005), Ca²⁺ channels in neurons (Norris et al. 2002), calcium levels in dendrites (Kuchibhotla et al. 2008), and welfare of dendritic spines (Wu et al.). All of these CaN-regulated events may impact memory function and their

contribution to promoting cognitive abnormalities induced by acutely administered A β oligomers remains to be established. Nonetheless, while we presently don't know if CaN activity is increased in both neurons and glia, our IHC studies show a marked reduction of pCREB in hippocampal neurons following A β oligomer injection, and this decrease depends in some fashion by over-activation of CaN as it is reversed by FK506. Thus, it is prudent to speculate that a neuronal CaN/pCREB signaling cascade is centrally involved in the cognitive deficits acutely induced by A β oligomers as reported in the present study.

Currently, we do not know how long after exposure to A β oligomers FK506 may be administered still exerting beneficial effects. Preliminary evidence indicates that a similar FK506 treatment paradigm failed to reverse FC memory impairments in 12 month old Tg2576 mice (Tagliatella and Dineley, unpublished observation). At this age, cognitive function is rapidly deteriorating and initial plaque deposition may be observed (Kawarabayashi et al. 2001). These observations suggest that as the impact of A β is protracted in time, permanent or CaN-independent changes underlying cognitive impairments occur. It is therefore prudent to reason that there may be a temporal window of opportunity during the early phases of symptomatic AD, where efficacy of CaN-inhibition may be maximal. Further studies are however needed to precisely address this issue.

In the present studies we did not observe an increase in PP1+PP2A combined activity in response to CaN up-regulation. This is somewhat puzzling in that, although not directly activated by Ca²⁺/calmodulin, PP1 activity is known to be indirectly promoted by activation of CaN through CaN-mediated dephosphorylation (inactivation) of the PP1 inhibitor dopamine- and c-AMP-regulated phosphoprotein, Mr 32,000 (DARPP-32) (Hemmings et al. 1984; Nishi et al. 2002). Indeed, we previously found a concomitant increase of CaN and PP1+PP2A activity in the hippocampus of behaviorally impaired Tg 2576 mice and this increase of PP1+PP2A activity was normalized by inhibition of CaN with FK506 (Dineley et al. 2007). One possible explanation for this inconsistency may be in the different time of exposure to A β (prolonged in the case of Tg2576 mice vs. acute in the present studies), although further detailed studies will have to clarify this point.

Growing evidence supports a role for A β oligomers in perturbing cognitive function through affecting synapse homeostasis and synaptic plasticity (Walsh and Selkoe 2007).

Interestingly, while we recently reported that oligomeric A β induces CaN activation and CaN-dependent cell deficits in *in vitro* and *ex vivo* neuronal models (Reese et al. 2008), many of the studies investigating the synaptic and dendritic toxicity of A β have noted an involvement of CaN. For example, inhibition of CaN prevents decrease in LTP in the hippocampus produced by treatment with aggregated A β (Chen et al. 2002) and blocks A β oligomer-induced increase of LTD (Li et al. 2009); the CaN inhibitor FK506 affects NMDA receptor trafficking at the synapse (Snyder et al. 2005) and blocks endocytosis of AMPA receptors induced by A β oligomers thus preventing synapses disruption (Zhao et al. 2009); retraction of dendritic spines caused by oligomeric A β is mediated by an NMDA receptor-associated signaling pathway involving CaN (Shankar et al. 2007) and CaN inhibition restores normal Ca²⁺ levels in dendrites in the CNS of APP transgenic mice *in vivo* (Sun et al. 2009).

These collective studies suggest that through mediating synaptic dysfunction, CaN may play an essential role in memory deficits acutely induced by A β oligomers, though no direct evidence of such an effect was yet available. Our current results show for the first time that inhibition of CaN indeed reverses deficits induced acutely by A β oligomers in a mouse model of fear conditioned memory recall, thus establishing *in vivo* a direct link between A β oligomers, CaN and cognitive function. Given recent evidence that CaN hyperactivation in the brain of AD patients inversely correlates with standardized cognitive test scores (Abdul

et al. 2009), our results further suggest that targeted inhibition of CaN in the CNS should be explored as a therapeutic opportunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Experimental Design

B. Contextual Fear Conditioning Memory Test

C. Cued Fear Conditioning Memory Test

D. Phosphatase activity in Hippocampus

Figure 1.

Acute icv injection of Aβ oligomers induces CaN activity and impairs FC memory in mice. A. A schematic illustrating the experimental design used in these experiments. Aβ oligomers (200 pmoles/3 μl/mice) were injected icv 24 hr prior to FC training (48 hr prior to FC test and sacrifice). Prior to FC training half of the mice were treated with FK506 (10 mg/kg ip) and half received saline.

B. Contextual FC test scores in mice treated with saline icv followed by saline ip (Saline/Saline) or Aβ icv followed by saline ip (Aβoli/Saline) or FK506 ip (Aβoli/FK506). Percent freezing (reflecting memory recollection) for each group is shown for each 60 sec block of the 300 sec test period (upper panel) or as cumulative data for the entire test period (lower panel). N=15 mice per group. **:p<0.01 vs. Saline/Saline or Aβoli/FK506 (ANOVA).

C. Cued FC test scores in the same mice showed in B. Percent freezing (reflecting memory recollection) for each group is shown for each 60 sec block of the 300 sec test period (upper panel) or as cumulative data for the entire test period (lower panel). Cue was presented at the end of the 120–180 sec block period. N=15 mice per group. No statistical difference was noted among groups within the pre-cue or post-cue freezing values.

D. Calcineurin activity (white column) and PP1/PP2A combined activity (black column) in the hippocampus of the same mice sacrificed at the end of the FC memory tests (contextual and cued). N=5 mice per group (randomly chosen from the 15 per group used in behavioral studies). *:p<0.05 vs. Saline/Saline or Aβoli/FK506 (ANOVA).

Figure 2A

Figure 2B

Figure 2.

Active (phosphorylated) neuronal CREB is reduced in a CaN-dependent fashion in the hippocampus of mice injected icv with A β oligomers.

A. Mice treated with saline icv followed, 18 hr later, by saline ip (Sal/Sal) or FK506 (10 mg/kg, Sal/FK506) or treated with A β icv (200 pmoles/3 μ l/mice) followed, 18 hr later, by saline ip (A β oli/Saline) or FK506 ip (A β oli/FK506). Mice were sacrificed 6 hr after the second injection (24 hr after the icv injection). Western blots detecting pCREB were performed on protein extracts from the hippocampus and the blots re-probed for total CREB. Band intensity was determined by a computer-assisted densitometry and mean values from 4 animals each group expressed as the ratio of pCREB/CREB (upper panel). Lower panel shows a representative Western blot from these experiments. *: $p < 0.05$ vs. Sal/Sal or A β oli/FK506 (ANOVA)

B. Representative confocal micrographs depicting NeuN (neuronal marker) and pCREB dual staining in the hippocampus (CA3 area) of mice injected icv with A β oligomers followed by ip FK506 as described in A above. Sections were mounted using a DAPI-containing mounting medium to visualize cell nuclei. Arrows point at neuronal cell bodies illustrating decrease of pCREB staining in the A β oligomer-injected mouse. Reference bars are 100 μ m long.

A. Experimental Design

B. LTP in CA1 neurons

C. Phosphatase activity

Figure 3. Inhibition of calcineurin reverses the impairment of long-term potentiation induced by oligomeric Aβ in hippocampal neurons

A. High-frequency synaptic stimulation (HFS)-induced expression of long-term potentiation (LTP) in the hippocampus of rat brain slices treated for 1 hr with vehicle (a) or with 0.5 μM oligomeric Aβ (b). Slices were additionally pretreated (45 min after addition of Aβ) with FK506 (10 μM, c), rapamycin (10 μM, d), or FK506 and rapamycin combined (e). Each symbol is the average of 10 monosynaptic excitatory postsynaptic currents (EPSCs). Peak amplitudes were measured and expressed as percent of baseline values before HFS.

Individual traces in inserts show averaged EPSCs recorded before and 30 min after HFS. Whole-cell patch-clamp recordings of individual hippocampal neurons were made in the CA1 region. Monosynaptic EPSCs were evoked by electrical stimulation (200 μs square-wave pulses at 0.033 Hz) of the Schaffer collateral/commissural pathway. LTP was evoked by 3 high-frequency trains (100 Hz, 1 s duration each; intertrain-interval 20 s). For illustration purposes, all scales were set to a maximum of 400 %.

B. Calcineurin activity (white columns) and PP1+PP2A combined activity (black columns) assayed in the same brain slices shown in A at the end of the LTP recording. N=3 per group; ***: $p < 0.001$ vs. control (ANOVA).