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Corticotropin-releasing factor receptor-1 antagonism mitigates beta amyloid pathology and cognitive and synaptic deficits in a mouse model of Alzheimer's disease

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Abstract

Introduction: Stress and corticotropin-releasing factor (CRF) have been implicated as mechanistically involved in Alzheimer's disease (AD), but agents that impact CRF signaling have not been carefully tested for therapeutic efficacy or long-term safety in animal models.

Methods: To test whether antagonism of the type-1 corticotropin-releasing factor receptor (CRFR1) could be used as a disease-modifying treatment for AD, we used a preclinical prevention paradigm and treated 30-day-old AD transgenic mice with the small-molecule, CRFR1-selective antagonist, R121919, for 5 months, and examined AD pathologic and behavioral end points.

Results: R121919 significantly prevented the onset of cognitive impairment in female mice and reduced cellular and synaptic deficits and beta amyloid and C-terminal fragment- β levels in both genders. We observed no tolerability or toxicity issues in mice treated with R121919.

Discussion: CRFR1 antagonism presents a viable disease-modifying therapy for AD, recommending its advancement to early-phase human safety trials.

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Keywords:

Alzheimer's disease; R121919; Corticotropin-releasing factor receptor; Corticotropin-releasing hormone; Hippocampus; Cognitive deficits; Synaptic deficits; Stress; Beta amyloid

1. Background

The neurodegenerative process in Alzheimer's disease (AD) is characterized by progressive accumulation of beta amyloid (A β) protein and hyperphosphorylated forms of tau protein, leading to synaptic dysfunction and cognitive

impairment. Recent work has implicated environmental factors, prominently including stress, as conferring susceptibility to AD pathogenesis [1]. In addition to data demonstrating that AD mouse models have perturbations in central stress signaling and display increased anxiety behavior [2–4], epidemiologic work demonstrates that individuals prone to experience psychological distress or anxiety are more likely to be diagnosed with AD than age-matched controls [5,6] and exhibit more rapid rates of cognitive decline [6].

Corticotropin-releasing factor (CRF) is best known as the hypothalamic neuropeptide initiates the endocrine stress response via the type 1 corticotropin-releasing factor

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receptor (CRFR1), a G protein-coupled receptor (GPCR) positively coupled to adenylate cyclase [7]. CRFR1 is also expressed widely in the brain, including AD-relevant regions as isocortex, hippocampus, and amygdala [8]. A substantial number of studies demonstrate a role for CRF and CRFR1 signaling on AD-related end points [4,9–13].

To assess the efficacy of CRFR1 antagonism on cognitive and pathologic end points, we used a double transgenic AD mouse model (PSAPP) that develops A β pathology in the cortex and hippocampus beginning at 3–4 months of age in both genders and cognitive impairment in females by 6 months of age [14,15]. We took advantage of data from recent clinical trials suggesting that anti-A β treatments may be effective in humans when administered at preclinical/predementia stages of AD (rather than after cognitive symptoms are present [16]) and used a preclinical prevention paradigm similar to that of current anti-A β AD prevention trials [17] to administer a second generation, small-molecule CRFR1 antagonist to groups of 30-day-old AD mice daily for 5 months. Using this strategy, we find that CRFR1 antagonism is a safe and viable disease-modifying treatment for AD.

2. Methods

2.1. PSAPP mice

An AD-Tg mouse model (B6.C3-Tg [APPswe, PSEN1dE9] 85Dbo/Mmjax, stock no. 004462) and wild type (WT) mice (C57BL/6J, stock no. 000664) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house. Male and female PSAPP mice, which contain a chimeric mouse/human *APP* gene co-expressed with a mutant human *PSI* gene, were used [14]. WT littermates were used as control. All mice were weaned at 21 days of age and entered the study at 30 days of age. Mice were housed (2–4 mice/cage) in a temperature-controlled room (22°C) with a 12-h light-dark cycle. A total of 102 mice, with individual group sizes per condition ranging from 11 to 15 mice were randomly assigned to either drug or vehicle arms based on gender and transgenic status. The UCSD Institutional Animal Care and Use Committee (IACUC) approved all experimental protocols.

2.2. R121919 administration

For pharmacologic blockade of CRFR1, we used the well-characterized, small-molecule CRFR1-selective antagonist, R121919 [18]. R121919 was dissolved in a vehicle solution composed of 0.3% tartaric acid and 5% vol/vol polyethoxylated castor oil. Vehicle solution was used as a control and administered as prepared previously without R121919. Both R121919 and vehicle solution were mixed by vortexer and sonicator to ensure a complete mixing. The final pH of the vehicle or R121919 was at pH 3. Mice were given subcutaneous injections of vehicle or R121919 (20 mg/kg/d) for 150 days. The 20 mg/kg/d was chosen

based on the efficacy of this dose to antagonize a variety of stress-related end points [12,13].

2.3. Morris water maze

The morris water maze (MWM) was used to test spatial learning and memory as a function of R121919 treatment. After basic training in the paradigm (visible platform), a probe test and spatial learning tasks were performed. Mice were given four 90-second trials per day for eight consecutive days. In the second spatial learning test, the platform was relocated into a new quadrant each day. For this task, mice were given four trials per day (90 seconds per trial) to search for the relocated platform and each mouse was released into the pool after 10 seconds of inter-trial interval (ITI) at the same start location. Testing involved placing each mouse in the tank at water-level, facing the pool wall, and at one of two start positions equidistant from the platform. Video tracking was initiated once the mouse was released and terminated automatically when the animal remained on the platform for >3 seconds. Mice were allowed to remain on the platform for a total of 10 seconds during the ITI.

2.4. Sample collection

After behavioral testing, mice were sacrificed under deep anesthesia with isoflurane, trunk blood was collected, and plasma and serum were frozen and stored at –80°C. Brains were rapidly removed after decapitation, and the right hemisphere cortex and hippocampus were harvested on ice for biochemical assays [12,13], whereas the left hemisphere was saved for immunohistochemical analyses. Livers were snap frozen and stored at –20°C for pathologic analyses.

2.5. Immunohistochemical analyses

For detection of diffuse and neuritic A β plaques, an N-terminal-specific anti-human A β monoclonal antibody (82E1) [19] and stereological methods [20] were used. To assess changes in cell and synaptic densities in the cortex and hippocampus, microtubule-associated protein (MAP2) and anti-synaptophysin antibodies were used. Details of immunohistochemical procedures, quantification, and stereological analyses are provided in supplemental methods.

2.6. Western blot

To analyze changes in both full-length amyloid precursor protein (APP) and C-terminal fragments (CTFs) of APP, 22C11 and CT-15 antibodies were used, respectively. A β peptides were detected with 82E1. Details of Western blot procedures and quantification are described in supplemental methods.

2.7. A β peptide analyses

For the purpose of A β peptide identification, samples were analyzed using an ABI 4800 matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS), followed by established protocols [21]. Levels of A β -38, -40, and -42 were detected using MesoScale-validated (MSD) triplex bioassays.

2.8. Enzymatic assays

β -Site APP cleaving enzyme 1 (BACE-1) activity was determined using enzymatic assay kits from Abcam (Cambridge, MA, USA). The hippocampal tissues prepared previously were used ($n = 10$ mice per group). The experimental procedures were followed as per the manufacturer's instructions. Relative fluorescence units were detected at $\text{Ex} = 345 \text{ nm}$ and $\text{Em} = 505 \text{ nm}$ by a SoftMax Pro 6.3 microplate reader from Molecular Devices (Sunnyvale, CA, USA).

2.9. Liver histopathologic and serum analyses

Liver histopathology and serum biochemistry were performed by Dr Kent Osborn in the Pathology Core of the Animal Care Program Diagnostic Laboratory at UCSD.

2.10. Corticosterone assays

Plasma corticosterone was detected using radioimmunoassay (RIA) kits from MP Biomedicals (Santa Ana, CA, USA) according to the manufacturer's instructions.

2.11. Statistical analyses

For all analyses, nonparametric t tests or analysis of variance in conjunction with Tukey's multiple comparison post-hoc tests were conducted using the GraphPad Prism 6.02 software. All data are expressed as the mean $\pm$ standard error of the mean. An α level of $P < .05$ was accepted as statistically significant.

3. Results

3.1. In vivo pharmacology and radioligand binding assays

Our prior work demonstrated that systemic administration of R121919 (20 mg/kg/d) was effective in blocking stress-induced tau phosphorylation [12,13]. To confirm that this dosage displayed target engagement upon chronic administration in AD mice, the ability of R121919 to disrupt binding of radio-labeled sauvagine, a peptide structurally related to CRF that binds both CRFRs was measured. Reduction in sauvagine binding in AD mice was observed at 20 mg/kg of R121919, whereas the lower dose (10 mg/kg) was much less effective (Supplementary Fig. 1).

3.2. Learning and memory performance

3.2.1. Baseline effects

The MWM was used to test spatial memory performance in AD and WT mice treated with R121919 or vehicle. During the visible platform portion of the test, both AD and WT mice had similar performance to find the platform regardless of treatment (vehicle vs. R121919), indicating that AD and WT animals have equivalent visual capabilities and R121919 did not impact visual ability (data not shown). During hidden platform testing (spatial learning test 1, Fig. 1A), female AD mice performed worse on the task compared with WT females ($P < .05$).

3.2.2. Spatial memory performance and retention (fixed platform)

In female AD mice, R121919 treatment prevented spatial memory deficits; R121919-treated female AD mice took less time to find the hidden platform compared with vehicle-treated female counterparts ($P < .05$). Significant treatment effects were also found for distance traveled, with R121919-treated female AD mice having traveled significantly shorter distances to locate the hidden platform (Fig. 1B). Importantly, cognitive performance in female AD mice treated with R121919 did not significantly differ from that of WT counterparts ($P > .05$).

R121919 effects on memory retention were examined using a probe test. In terms of performance, drug-treated female AD mice spent a greater percentage of time in the target quadrant, compared with vehicle-treated female AD mice ($P < .05$, Fig. 1C). As seen with spatial memory task 1, drug-treated female AD mice had equivalent percent time in the target quadrant compared with other those of the WT group ($P > .05$, Fig. 1C).

3.2.3. One-trial spatial memory (movable platform)

To confirm the treatment effects seen, we used a second cognitive test using a one-trial spatial memory task. This task was administered immediately after completion of the fixed platform tasks and required the animals to find a platform that was relocated to different quadrants. In the training trial, animals were kept on the platform for 10 seconds and then immediately restarted for testing. In this task, we again found a significant effect of R121919 treatment on performance (both time spent and distance traveled) in female AD mice, with drug-treated cohorts requiring less distance to complete the task compared with their vehicle counterparts ($P < .05$, Fig. 2B).

3.2.4. Cognitive performance in male mice

In line with reports using this AD model, we observed no significant difference in performance (time spent and distance traveled) for male AD mice compared with male WT cohorts. We also observed no impact of R121919

Fig. 1. Performance during spatial learning test 1 and the probe test. (A) Time, (B) distance traveled, and (C) percent time spent in each quadrant during the probe test. All values are expressed as mean $\pm$ SEM, $n = 10$ mice per group. Genotype effect: $+P < .05$, $++P < .01$ versus WT/AD. Treatment effect: $*P < .05$, $**P < .01$, and $***P < .001$ versus AD vehicle/AD drug. Abbreviations: SEM, standard error of the mean; AD, Alzheimer's disease.

treatment on cognitive performance in male AD mice (Supplementary Fig. 2).

Overall, these results demonstrate that R121919 significantly prevented/delayed spatial learning and memory deficits in female AD mice and made them indistinguishable from their female WT counterparts. Furthermore, in no task did we observe an impact of R121919 in animals that did not exhibit cognitive impairment (e.g., WTs or male AD mice), which suggests that R121919 has no impact on memory function in the absence of a deficit.

3.3. Mechanisms underlying R121919 action

3.3.1. Stereological assessment of A β load

Our behavioral data demonstrate that R121919 can prevent onset of cognitive impairment in female AD mice.

Because our overarching hypothesis posits that measurable deficits in cognition are preceded by a lengthy preclinical phase characterized by damage to cellular/synaptic networks as a result of A β accumulation [22,23], we examined cognitive effects associated with changes in A β pathology. To determine whether R121919 could reduce A β accumulation, we quantified the percent area occupied by plaques in AD mice as a function of treatment. Using a N-terminal-specific anti-human A β monoclonal antibody (82E1) and stereological methods (see Supplementary Methods), we confirmed that AD mice of both genders had accumulation of A β plaques in the hippocampus and cortex in line with previous reports of this mouse model at 6 months of age (Fig. 3, and [14,15]). In terms of drug effects, both male and female AD mice treated with R121919 had significantly reduced accumulation of A β in both the hippocampus (male,

Fig. 2. Performance during the one-trial learning test. (A) Time and (B) distance traveled during the one-trial learning test. AD-female mice treated with R121919 had better performance with less time and a shorter distance, which demonstrates that treatment with CRFR1 antagonism prevented short-term memory deficits. All values are expressed as mean $\pm$ SEM, $n = 10$ mice per group. The genotype effect: $+P < .05$ versus WT/AD. Treatment effect: $*P < .05$, $**P < .01$, and $***P < .005$ versus AD vehicle/AD drug. Abbreviations: AD, Alzheimer's disease; CRFR1, corticotropin-releasing factor; SEM, standard error of the mean.

Fig. 3. Effect of drug treatment on AD mice treated with vehicle (left) or drug (right) on Aβ plaque load. (A) Aβ plaques were labeled in 6-month-old vehicle or drug-treated AD mice using antibody 82E1 (grayscale, hemisphere, first row). (B) Aβ plaque load (%) of the cortex/hippocampus was determined (second row). The percentage of area covered by Aβ plaques in both male and female AD and WT mice treated with vehicle or R121919 was quantified using stereology. Both diffuse and neuritic plaques were identified using antibody 82E1. In the hippocampus, reduced plaque load (%) was observed in both male ($P < .05$) and female ($P < .01$) AD mice treated with R121919. Untreated (vehicle) mice had higher plaque load (%) in both male ($P < .01$) and female ($P < .05$) AD mice compared with their WT counterparts. All values are expressed as mean $\pm$ SEM, $n = 10$ mice per group. The treatment effect: * $P < .05$, ** $P < .01$ versus vehicle/drug. Scale bar indicates 250 μ m. Abbreviations: AD, Alzheimer's disease; Aβ, beta amyloid; SEM, standard error of the mean.

$P < .05$; female $P < .01$; Fig. 3B) and cortex, compared with vehicle-treated cohorts (male, $P < .05$; female $P < .05$; Fig. 3B). These data demonstrate that R121919 can prevent or delay accumulation of Aβ, which may be an important mechanistic step for dendritic and synaptic loss and eventual cognitive impairment in these mice.

3.3.2. Synaptic and dendritic deficits

Synaptic loss is best correlated to cognitive impairment in humans with AD [24] and AD transgenic mice [22,23]. We, therefore, hypothesized that R121919 effects on Aβ accumulation would be associated with mitigation of synaptic and dendritic deficits seen in AD mice. A significant reduction in the percent area-containing axon terminal (synaptophysin) and dendritic (MAP2) labeling was observed in both frontal cortex and hippocampus between female AD mice (cognitively impaired) and their WT counterpart mice ($P < .05$, Fig. 4A–D). This loss was rescued by R121919 treatment to the extent that we observed no difference between the WT-vehicle and AD-drug groups ($P > .05$, Fig. 4A–D). In contrast to female AD mice, and in line with the lack of cognitive impairment, male AD mice displayed no significant difference in dendritic staining in either the hippocampus or cortex compared with WT cohorts, nor were any effects of R121919 treatment observed ($P > .05$, Fig. 4E and F). We observed a small but significant reduction in synaptophysin labeling in the cortex of male AD mice compared with their WT counterparts, which was prevented by R121919 treatment (Fig. 4G–H). Collectively, these data

provide support for CRFR1 antagonism as a disease-modifying treatment, and the dramatic reductions in synaptic and dendritic immunoreactivity seen with cognitive impairment in female AD mice were prevented with R121919 treatment. In asymptomatic transgenic mice (males), R121919 was capable of preventing small reductions in synaptic density.

3.3.3. Aβ pathways

Biochemical analyses were used to measure levels of APP-CTF-α and CTF-β in hippocampal extracts from male and female AD mice using Western blot (Fig. 5A). Significantly reduced APP-CTFs (α, $P < .005$; β, $P < .001$) were detected in both male and female AD mice treated with R121919 compared with vehicle (Fig. 5C and D). To better understand the effect of R121919 on APP-CTFs in female Tg brain tissues, BACE-1 activity in the hippocampi of female AD mice was measured and analyzed. There were no differences in BACE-1 activity of the cortical tissues ($n = 10$ mice per group, data not shown). However, a significant decrease in BACE-1 was found in the female AD mice treated with drug compared with their vehicle counterparts (Fig. 5D) ($n = 14$ mice per group), indicating that R121919 may have the impact on the levels of BACE-1 activity in our AD mouse model. Previous studies have reported that increased BACE-1 activity can elevate APP-CTF levels, which may lead to cognitive decline [25]. Here, we have also demonstrated that decreased

Female

Fig. 4. Impact of R121919 on synaptic and dendritic deficits seen in AD mice. Comparison of MAP2-positive dendritic structures in the frontal cortex and hippocampus of female (A) and male (E) WT and AD mice. Quantifications of percent area of the frontal cortex and hippocampus covered by MAP2-immunoreactive dendrites of female (B) and male (F) WT and AD mice. (C) Changes in synaptophysin and synapse staining in the frontal cortex and hippocampus of female (C) and male (G) WT and AD mice. Quantitative assessments of percent area of the frontal cortex and hippocampus covered by synaptophysin and synapses of female (D) and male (H) WT and AD mice. All values are expressed as mean $\pm$ SEM, $n = 5$ mice per group. The genotype effect: $+P < .05$ versus WT/AD. Treatment effect: $*P < .05$ versus vehicle/drug. Scale bar = 15 μ m. Abbreviations: AD, Alzheimer's disease; SEM, standard error of the mean.

APP-CTFs levels were closely correlated to reduced BACE-1 activity levels in female AD mice treated with drug, suggesting that this reduction of APP-CTFs was because of the decrease of BACE-1 activity.

3.3.4. A β peptides

To study whether changes in A β peptides were also altered as a function of R121919 treatment, A β -38, -40, and -42 were analyzed using validated bioassays. As other studies expected, levels of A β peptides (38, 40, and 42) were significantly increased in AD mice compared with WT mice, although no change was found in levels of A β peptides as a function of R121919 treatment ($P > .05$, Fig. 6). We also found no significant differences in A β peptides with R121919 treatment using either Western blot or MALDI-TOF/TOF-MS.

3.4. R121919 tolerability and safety

3.4.1. Liver morphology and biochemistry

To determine whether chronic treatment with R121919 was well tolerated and safe, we monitored levels of liver enzymes in AD mice (UCSD Pathology Core). Levels of blood urea nitrogen, albumin, cholesterol, total bilirubin, alanine transaminase, and aspartate transaminase were analyzed in serum samples from male and female WT and AD mice treated with vehicle or drug. No effect of R121919 was observed on any end point (Supplemental Fig. 3A–F).

Liver morphology was investigated to complement serum biochemistry. Histopathologic assessments revealed that both vehicle- and drug-treated mice were normal, with no signs of toxicity present (Supplemental Fig. 3G and H).

3.4.2. Grooming and weight gain

Animals were monitored daily for grooming behavior and body weight (Supplemental Fig. 4). Grooming behavior was assessed daily by visual inspection of fur and skin. R121919-treated cohorts (both WT and AD) were indistinguishable from vehicle-treated cohorts in terms of grooming and appearance (data not shown). In terms of body weight, all mice were weighed daily each morning. We found that no significant differences were observed with R121919 or vehicle treatment in female WT mice. Conversely, a small but statistically significant effect of R121919 was observed on weight gain in male WT mice (5 and 6 month, each $P < .05$ compared with vehicle, Supplemental Fig. 4A). A similar reduction was observed in male AD mice, which was detectable beginning at 3 months of age (Supplemental Fig. 4B). Drug-treated female AD mice also had a small but significant reduction in weight gain, compared with vehicle-treated cohorts only at the 5-month time point (Supplemental Fig. 4C and D).

3.4.3. Glucocorticoids

We used RIAs to determine the impact of long-term R121919 treatment on plasma corticosterone levels. No significant changes ($P > .05$) were seen in basal levels of

Male

Fig. 4. (continued).

plasma corticosterone in WT or AD animals as a function of R121919 treatment (Supplemental Fig. 5B) as previously reported (e.g., [26]).

4. Discussion

To our knowledge, this study is the first to examine the potential of CRFR1 antagonism as a preventative therapeutic for AD and demonstrate that chronic administration of a CRFR1 antagonist presents a safe and effective treatment in this context. We find that such a chronic treatment regimen can delay the onset of cognitive impairment and rescue synaptic and dendritic deficits in a mouse model of AD. In terms of pathology, AD mice receiving treatment had greatly reduced accumulation of A β plaques and concomitant reduction in APP CTF- β , suggesting that the upstream mechanisms involve modulation of A β generation pathways. These data are consistent with the notion that CRFR1 antagonism can have direct actions on A β production and support previous work demonstrating direct regulation of secretase enzymes via GPCRs [27]. Although male AD mice had similar regulation of A β accumulation with R121919 treatment, but have a longer clinical asymptomatic period compared with their female counterparts, we were able to assess the impact of CRFR1 antagonism on pathology and cognitive change in parallel. In support of studies showing that synaptic and dendritic changes are better correlated with cognitive decline than pathology [24], we too found that male mice (clinically asymptomatic) had very small reductions in synaptic staining and no changes in dendritic staining, indicative of the prepathologic stage of AD. As a whole, our data support the hypothesis that interference of CRFR1 signaling is a safe and effective disease-modifying treatment for AD.

4.1. Implications for AD neuropathology

Although age is the primary risk factor for developing AD, stress is also implicated, and a number of studies have attempted to address the mechanisms by which stress exposure and/or sensitivity may contribute to pathogenesis. Glucocorticoids (corticosterone in mice, cortisol in humans) are dominant stress hormones that are increased in humans with AD and linked to neuropathology and neuronal vulnerability. Although glucocorticoids would appear to be as an important target in AD, steroid treatment in human AD trials does not alter cognition (Alzheimer's Disease Cooperative Study Prednisone Trial [28]) and results of studies attempting to draw mechanistic links to pathology and cognition in AD rodent models have been mixed [3,4,9,29–32]. Furthermore, in contrast to the effects seen with genetic ablation of CRFR1 [12,33], small-molecule CRFR1 antagonists are lipophilic, have primarily central actions [18,34], and minimally impact basal levels of glucocorticoids or the ability to mount a stress response [26,34–36]. Mechanistic studies suggest that small-molecule CRFR1 antagonists can induce central changes in nuclear translocation of glucocorticoid and mineralocorticoid receptors in a stress-independent manner [37]. The data presented here provide further support for the hypothesis that interference of stress steroids may not be critical for obtaining clinical and pathologic benefits in AD.

4.1.1. Role of the CRF system in AD

Anatomic and biochemical data indicate the involvement of CRF in the development of AD. For example, reduced cortical CRF immunoreactivity (in the face of increased hypothalamic expression) is a prominent neurochemical

Fig. 5. Impact of R121919 on APP processing in AD mice. (A) Hippocampi from AD mice (vehicle vs. drug) were homogenized in RAB buffer and separated using a 10%–20% gradient gel. CTFs (CTFα and CTFβ) were detected by the CT-15 antibody. Full-length APP was probed using the 22C11 monoclonal antibody. β-Actin served as a loading control. (B) Levels of CTF-α/APP were significantly reduced in the hippocampus of male and female AD mice treated with drug (***P* < .005). (C) Levels of CTF-β/APP were significantly reduced in the hippocampus of AD Tg male and female mice treated with drug (*****P* < .001). (D) A significant decrease of bsite APP-cleaving enzyme-1 (BACE-1) activity was observed in the hippocampus of female AD mice treated drug (**P* < .05) compared to vehicle treated cohorts. All values are expressed as mean ± SEM, *n* = 3 mice/group. Abbreviations: APP, amyloid precursor protein; AD, Alzheimer's disease; CTF, C-terminal fragment; V, vehicle; D, drug.

change in AD [38,39], which occurs early in disease progression, and is focused in areas vulnerable to AD neuropathology [40–43]. CRF-positive dystrophic neurites have been found associated with Aβ plaques [41] and marked increases in CRF binding have been described in specific cortical regions of AD patients, suggesting upregulation of CRFRs in impacted areas [44]. Furthermore, studies in rodent models demonstrate that CRF overexpression can lead to tau phosphorylation and aggregation, brain atrophy, and cognitive impairment [10,11,13,45].

Evidence from our laboratory and others demonstrates that overexpression of CRF or exposure to chronic stress in rodents can induce phosphorylation and solubility changes in the microtubule-associated protein, tau, a process that is reliant on CRFR1 [10,12,13,33]. Furthermore, exposing rodents to chronic emotional stress results in increased phosphorylation and decreased solubility of the tau protein; changes that are also strictly dependent on CRFR1 signaling [12,33]. In addition to work on tau, several reports demonstrate that CRF or stress exposure can impact Aβ production and accumulation in AD models [9,11,46,47] and that stress-induced Aβ plaque formation in adult AD mice can be reduced by CRFR1 antagonism [4]. In particular, our recently published work

demonstrates that genetic ablation of CRFR1 greatly reduces the production of APP CTFs and accumulation of Aβ in the brains of AD mice [47]. Regardless of these demonstrated effects, the data presented here and in these studies leave open to questions why signaling through a central neurotransmitter/neuroendocrine peptide receptor would be relevant in affecting disease-related end points. Although it is possible that CRFR1 antagonists impact targets other than CRFR1, the fact that mechanistic studies have identified specific pathways involved in CRFR1 antagonism [48] and that the effects seen pharmacologically are consistent with CRFR1 knockout studies makes this possibly unlikely [12,33].

4.1.2. Reduction in Aβ accumulation without reduction in Aβ peptides?

Aβ is derived from APP, by the action of two aspartyl proteases, β- and γ-secretases. Resultant fragments of APP are left behind in the membrane after proteolysis, called CTFs. β-Secretase (BACE-1) cleaves APP, generating the C99 fragment, which is also called CTF-β and comprises the N-terminus of Aβ. When γ-secretase cleaves CTF-β, Aβ is released and is found in several forms, the most abundant of which consist of 38, 40, or 42 amino acids. An alternative,

Fig. 6. Quantification of A β peptides using MesoScale bioassays. RAB (left) and RIPA (right) extracts. (A) Cortical A β -38 levels. (B) Cortical A β -40 levels. (C) Cortical A β -42 levels. (D) Cortical A β -40/42 ratios. All values are expressed as mean $\pm$ SEM, $n = 10$ mice per group. Abbreviations: A β , beta amyloid; SEM, standard error of the mean; V, vehicle; D, drug; M, male; F, female.

nonamyloidogenic pathway, also exists and involves a third protease, α -secretase. Unlike BACE-1, α -secretase cuts within the A β domain of APP, generating the CTF- α fragment (also known as C83). We found that the levels of CTF- β were significantly reduced with R121919 treatment in AD mice. Interestingly, although both A β plaque accumulation and CTF- β levels were reduced, as were CTF- β levels, we were not able to find significant changes in either A β 40 or

A β 42 using immunoblot, MALDI-TOF/TOF-MS, or MSD bioassay. With our anatomic data demonstrating low levels of A β accumulation as a function of drug treatment (Fig. 3), our initial reaction was that the absolute levels of these peptides were below the detection threshold for the assays due to the early stage of the disease (cf [4]). Although our hypothesis may be correct, data sets support disconnect between dramatic therapeutic effects on A β accumulation and absolute change in A β peptides with chronic treatments in mature stage AD mice, including the PSAPP mouse model used in our study [49,50]. The mechanisms underlying this disconnect may relate to the ability of current reagents to detect non-aggregated A β peptides (e.g., [49]). For example, selective detection of oligomeric A β peptides has been reported with the presence of the lower molecular weight species being undetectable [51]. Therefore, we believe that the lack of change in A β peptides observed here may be due to the low levels of disaggregated A β that exist in our animals at this early stage or the detection limit of techniques.

4.2. Moving forward and next steps

4.2.1. Is R121919 a viable candidate for translation?

Owing to the fact that stress may precipitate or worsen a host of central nervous system and systemic pathologies, substantial basic and clinical effort attention has been directed toward developing drugs that target the CRF system, with a particular focus on CRFR1 antagonists. Although clinical trials of R121919 have found isolated instances of liver enzyme elevation [52], we did not observe this in our rodent model nor has been reported in other studies [4]. As detailed in the results section, the change in weight gain we observed was isolated and not associated with any pathologic findings. This observation is also mitigated by the fact that AD animals have a tendency to be overweight compared with their WT counterparts. Regardless of our and other preclinical data demonstrating safety and tolerability of chronic R121919, repurposing this drug for AD human studies is likely not possible. We are, therefore, actively developing high throughput screening assays to facilitate the identification and validation of new CRFR1 antagonists.

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Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jalz.2015.09.007>.

RESEARCH IN CONTEXT

1. Systematic review: Current Food and Drug Administration-approved drugs do not prevent, modify, or reverse Alzheimer's disease (AD) and provide only modest and temporary symptomatic benefits. We tested whether the small-molecule, corticotropin-releasing factor receptor (CRFR)1-selective antagonist, R121919, could impact pathologic and behavioral end points in AD mice to determine whether this therapeutic approach could serve as a disease-modifying treatment for AD.
2. Interpretation: We demonstrate that chronic administration of the CRFR1 antagonist, R121919, to be well tolerated and efficacious as a disease-modifying therapy for AD.
3. Future directions: We recommend advancement of the CRFR1 antagonism program to early-phase safety trials for AD. In terms of basic science work, additional studies are needed to understand the mechanisms of CRFR1 antagonism effects on AD neuropathology. Studies in our laboratory center on the role CRFR1 antagonism may play on beta amyloid enzymes, inflammatory pathways, bioenergetics, and modulation of protein/DNA modifications induced by oxidative stress.

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