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Authors

Park, Hyo-Jin
Ran, Yong
Jung, Joo In
et al.

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The stress response neuropeptide CRF increases amyloid-β production by regulating γ-secretase activity

Hyo-Jin Park[1,2], Yong Ran[1], Joo In Jung[1], Oliver Holmes[3], Ashleigh R Price[1], Lisa Smithson[1], Carolina Ceballos-Diaz[1], Chul Han[4], Michael S Wolfe[3], Yehia Daaka[5], Andrey E Ryabinin[6], Seong-Hun Kim[2], Richard L Hauger[7], Todd E Golde[1,*] & Kevin M Felsenstein[1,**]

Abstract

The biological underpinnings linking stress to Alzheimer's disease (AD) risk are poorly understood. We investigated how corticotrophin releasing factor (CRF), a critical stress response mediator, influences amyloid-β (Aβ) production. In cells, CRF treatment increases Aβ production and triggers CRF receptor 1 (CRFR1) and γ-secretase internalization. Co-immunoprecipitation studies establish that γ-secretase associates with CRFR1; this is mediated by β-arrestin binding motifs. Additionally, CRFR1 and γ-secretase co-localize in lipid raft fractions, with increased γ-secretase accumulation upon CRF treatment. CRF treatment also increases γ-secretase activity *in vitro*, revealing a second, receptor-independent mechanism of action. CRF is the first endogenous neuropeptide that can be shown to directly modulate γ-secretase activity. Unexpectedly, CRFR1 antagonists also increased Aβ. These data collectively link CRF to increased Aβ through γ-secretase and provide mechanistic insight into how stress may increase AD risk. They also suggest that direct targeting of CRF might be necessary to effectively modulate this pathway for therapeutic benefit in AD, as CRFR1 antagonists increase Aβ and in some cases preferentially increase Aβ42 via complex effects on γ-secretase.

Keywords β-arrestin; γ-secretase; amyloid-β; corticotrophin releasing factor; stress

Subject Categories Neuroscience

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Introduction

Many insights into the pathophysiologic mechanisms leading to Alzheimer's disease (AD) have come from the study of familial forms of AD (fAD) (Golde *et al*, 2011; Goate & Hardy, 2012). Mutations in the amyloid β precursor protein (*APP*), Presenilin (*PSEN1* and *PSEN2*) genes, linked to fAD and studies demonstrating that they alter amyloid-β (Aβ) production or the properties of Aβ that promotes its aggregation, have provided pivotal support for the AD amyloid hypothesis (Hardy & Selkoe, 2002). Given extensive overlap of clinical and pathological phenotypes between fAD and sporadic late-onset AD (LOAD), it is generally accepted that pathophysiologic cascades are similar. Recent efforts to elucidate the genetic risk for LOAD are providing additional insights for AD pathogenesis; however, non-genetic factors are also important contributors to LOAD (Mayeux & Stern, 2012). As non-genetic factors may be more amenable to intervention than heritable aspects of the disease, it is important to establish what non-genetic factors contribute to AD risk and the biological basis for how these factors contribute to that risk.

Life style factors such as stress, diet, and physical and mental exercise may contribute to the risk of developing AD, or perhaps more accurately dementia (Mayeux & Stern, 2012). Multiple reports suggest that high chronic stress, or individuals with posttraumatic stress disorder or major depression, two affective illnesses linked to corticotropin-releasing factor (CRF) receptor (CRFR) dysregulation (Hauger *et al*, 2006, 2009), have a greater risk of developing AD (Wilson *et al*, 2003, 2005; Byers & Yaffe, 2011). Excessive activation of the hypothalamic–pituitary–adrenal (HPA) stress axis has also been correlated with the rate of AD progression (Csernansky *et al*, 2006). Links between stress and AD are also found in various animal models. Acute restraint, chronic isolation, and social stress exacerbate Aβ accumulation in *APP*

1 Center for Translational Research in Neurodegenerative Disease, Department of Neuroscience, McKnight Brain Institute, College of Medicine, University of Florida, Gainesville, FL, USA
2 Department of Pharmacology and Therapeutics, College of Medicine, McKnight Brain Institute, University of Florida, Gainesville, FL, USA
3 Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA
4 Department of Aging and Geriatric Research, College of Medicine, University of Florida, Gainesville, FL, USA
5 Department of Anatomy and Cell Biology, College of Medicine, University of Florida, Gainesville, FL, USA
6 Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA
7 Center of Excellence for Stress and Mental Health, Department of Psychiatry, VA Healthcare System, University of California, San Diego, CA, USA
*Corresponding author. Tel: +1 352 273 9456; E-mail: tgolde@ufl.edu
**Corresponding author. Tel: +1 352 294 5308; E-mail: kfelsenstein0@ufl.edu

mouse models (Dong *et al*, 2004; Jeong *et al*, 2006; Carroll *et al*, 2011; Huang *et al*, 2011; Rothman *et al*, 2012). Stress has also been reported to exacerbate tau pathology (Rissman *et al*, 2007; Carroll *et al*, 2011). However, the detailed molecular mechanism(s) by which stress modulates Aβ levels have not been elucidated.

Upon stress, hypothalamic paraventricular neurons release CRF into the portal circulation. CRF then binds to and activates the CRF receptor type 1 (CRFR1) on anterior pituitary corticotropic cells stimulating secretion of adrenocorticotropic hormone (ACTH), which then triggers adrenal glucocorticoid secretion (Vale *et al*, 1981; Rivier & Vale, 1983; Hauger *et al*, 2006). In addition to HPA regulation, defensive behavior, autonomic, metabolic, immune, and cardiovascular responses during stress are coordinated by the interplay of neuronal CRF and the related urocortin peptides (UCN1, UCN2, UCN3) binding and activating CRFR1 and CRFR2. Expression of CRFR1 is high in anterior pituitary corticotropic cells and widespread throughout the central nervous system, whereas CRFR2 is more discretely distributed in select forebrain neurons and peripheral tissues (Potter *et al*, 1994; Chalmers *et al*, 1995; Van Pett *et al*, 2000). Although elevated glucocorticoid levels are associated with cognitive symptoms of dementia (Csernansky *et al*, 2006; Lee *et al*, 2008), the involvement of a glucocorticoid receptor-dependent pathway remains controversial.

CRFR1 belongs to the Class B1 group of the G protein-coupled receptor (GPCR) superfamily. So far, CRFR1 and CRFR2 have been shown to preferentially couple to G_s proteins, leading to the stimulation of adenylate cyclase, although CRF receptors can also signal by activating the Gq-coupled PKC pathway and MAP kinase cascades (Dautzenberg & Hauger, 2002). CRFR1 signaling has been shown to increase interstitial fluid levels of Aβ40 in APP transgenic mice through an unknown mechanism, suggesting that CRF may be a causal factor in stress-induced Aβ accumulation and tau phosphorylation, and CRFR2 signaling has not been found to trigger regulate either APP processing or tau phosphorylation (Kang *et al*, 2007; Rissman *et al*, 2007, 2012).

In this study, having first validated and confirmed the effects of restraint stress on Aβ levels in non-transgenic C57BL/6J mice, possible roles of CRF and CRFR1 in the alteration of Aβ levels and γ-secretase trafficking were examined utilizing human and mouse neuronal cells. Data demonstrate that CRF not only increases Aβ through a CRFR1-dependent interaction with γ-secretase mediated by β-arrestin binding motifs in CRFR1, but also through a direct effect on γ-secretase. Additionally, we find that CRFR1 antagonists also differentially increase Aβ and mediate CRFR1 internalization. Further, we show that a CRFR1 antagonist antalarmin can act as an inverse γ-secretase modulator (iGSM) and fails to block stress-induced increase in brain Aβ despite inhibiting Gs-coupled CRFR1 signaling. The data provide insight into the biological basis for stress elevating the risk of AD mediated through increased production of Aβ by γ-secretase. In addition, these data indicate that current CRH antagonists may promote, rather than suppress, amyloid pathology.

Results

Restraint stress increases the levels of brain Aβ in C57BL/6J mice

To examine whether stress can increase brain Aβ in wild-type mice, C57BL/6J mice were subjected to acute restraint stress for 3 h and euthanized immediately or 24 h after the restraint session. Endogenous forebrain Aβ levels were analyzed using sandwich ELISA assays specific to Aβ40, Aβ42 and Aβ38 (Lanz & Schachter, 2008). After acute stress, Aβ levels increased compared to control group (Fig 1A). 24 h after acute stress, the increase is returned to baseline. APP and PS1 levels did not change following restraint stress compared with control groups (Fig 1B). Primary neuronal cells from neonatal C57BL/6J forebrains were also treated with CRF, and the levels of endogenous-secreted Aβ were measured. Consistent with a previous report (Kang *et al*, 2007), CRF increased the levels of Aβ40 (34%), Aβ42 (52%), and Aβ38 (11%) (Fig 1C).

CRF increases the levels of Aβ in human neuroblastoma SH-SY5Y cells

To further explore mechanisms by which CRF increases Aβ levels, we utilized human neuroblastoma cells, SH-SY5Y, which endogenously express the CRFR1 receptor (Supplementary Fig S1) (Schoeffter *et al*, 1999). Cells were treated with CRF, and Aβ levels in the conditioned media were measured. CRF treatment significantly increased production of Aβ40, Aβ42, and total Aβ (Fig 1D). An ~1.5-fold increase in the Aβ42:Aβ40 ratio to a magnitude similar to that observed with fAD linked APP and PSEN mutations (Borchelt *et al*, 1997). Levels of secreted sAPP, immature and mature APP, as well as APP C-terminal fragments (APP-CTFα and APP-CTFβ), were not significantly altered (Supplementary Fig S2A and B). As γ-secretase determines the ratios of Aβ42 to Aβ40, increases in Aβ suggest that CRF might regulate Aβ production through γ-secretase. However, no differences in the level of the γ-secretase subunits were detected (Supplementary Fig S2A and B). We also examined whether a glucocorticoid had any effect on Aβ levels (Landfield *et al*, 2007); however, corticosterone treatment did not alter Aβ, APP, or APP-CTF (Supplementary Fig S2C and D).

CRF treatment induces internalization of CRFR1 and γ-secretase

Interaction of an agonist with a GPCR promotes endocytosis of the ligand–receptor complex (Lefkowitz, 1998; Pitcher *et al*, 1998). To examine CRFR1 endocytosis, SH-SY5Y cell lines that constitutively expressed CRFR1 with a FLAG epitope tag were generated and modified surface biotinylation experiments performed. CRF treatment significantly increased the internalization of the CRFR1 receptors without altering total CRFR1 levels (Fig 2A).

Receptor internalization was also examined using a green fluorescent protein (GFP)-labeled CRFR1 expression construct and localization of the transfected CRFR1-GFP in HEK293 cells analyzed by confocal microscopy (Fig 2B). Prior to treatment, CRFR1 is primarily localized on the plasma membrane; after 1 h treatment, CRFR1-GFP is redistributed to intracellular vesicular compartments; primarily early endosomes (Fig 2B).

To examine whether CRF affects the intracellular localization of γ-secretase, CRFR1-GFP was expressed in N2a cells stably overexpressing all four γ-secretase components, anterior pharynx-defective 1 (APH1), Nicastrin (NCT), presenilin enhancer protein 2 (PEN2), and PSEN1 ("ANPP" cells) (Kim *et al*, 2003). Cell surface NCT, a

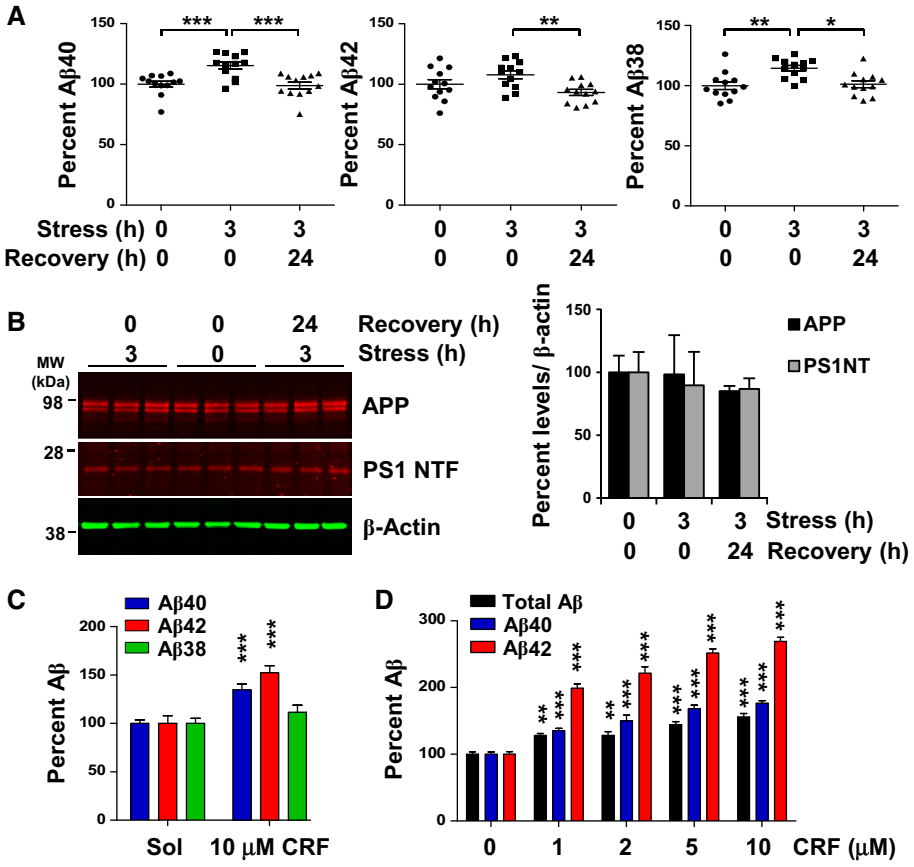

Figure 1. Acute restraint stress and CRF increased endogenous Aβ in C57BL/6J mice forebrains.

A Restraint stress increases the levels of Aβ40 by 15.2%, Aβ42 by 7.8%, and Aβ38 by 14.5%. Twenty-four hours after stress induction Aβ levels decreased to basal levels (n = 12/group, 13–14-week-old 18 males and 18 females were used).

B Restraint stress does not change APP and PS1 levels normalized to β-actin levels (n = 3/stress-induced group, n = 6/no stress group).

C CRF treatment increases the levels of Aβ40, Aβ42, and Aβ38 in primary cultures from P0 mouse brain (n = 6/group).

D CRF treatment increases the levels of Aβ40, Aβ42, and total Aβ in human neuroblastoma SH-SY5Y cells (n = 6/group).

Data information: Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.005 by one-way ANOVA and Tukey post-test (A, D) or t-test (C). The experiments were repeated twice (A, B) or three times (C, D).
Source data are available online for this figure.

marker for the mature γ-secretase complex, was labeled using a NCT antibody. Prior to treatment, CRFR1 largely co-localized with NCT on the plasma membrane (Fig 2C). Upon CRF treatment, cell surface CRFR1 and NCT decreased and redistributed to intracellular vesicles where they showed a high degree of co-localization (Fig 2C, enlarged, arrowheads).

CRFR1 associates with γ-secretase through β-arrestin binding motifs

The nature of the association of CRFR1 and γ-secretase was investigated by co-immunoprecipitation studies. Using conditions that preserve the γ-secretase complex in a functional state (Fig 3A, left panel, lane 4), a fraction of NCT, PS1, and PEN2 co-immunoprecipitated with CRFR1 (Fig 3A, left panel, lane 3). CRFR1 could also be co-immunoprecipitated with PS1 or PEN2 antibodies (Fig 3A, right panel, lanes 3 and 4). Mature NCT was enriched in both CRFR1- and PS1-precipitated lanes (Fig 3A, left panel, lanes 3 and 4), indicating that CRFR1 interacts with the active, γ-secretase complex (Kimberly *et al*, 2002).

It has been recently shown that G protein-coupled receptor 3 (GPR3) and the β2-adrenergic receptor mediate effects on γ-secretase and Aβ generation through β-arrestin2 (Thathiah *et al*, 2013); loss of β-arrestin2 reduces Aβ by decreasing γ-secretase cleavage. We investigated whether this mechanism might be responsible for CRFR1 regulation of γ-secretase activity and Aβ generation. We utilized CRFR1 mutants that alter interactions with β-arrestin. Serine and threonine residues in the C-terminal region and a serine-rich sequence in third intracellular loop (IC3) are potential sites for phosphorylation. Important motifs in the C-terminus include a potential arrestin binding site (T399-S400-P401-T402) and a class I PDZ binding domain (S412-T413-A414-V415) that may regulate CRFR1 interactions with signaling proteins (Fig 3B) (Oakley *et al*, 2007). Co-immunoprecipitation experiments show that the association of endogenous PS1 and the CRFR1 Δ386/IC3 and Δ386 mutants was reduced (Fig 3C)

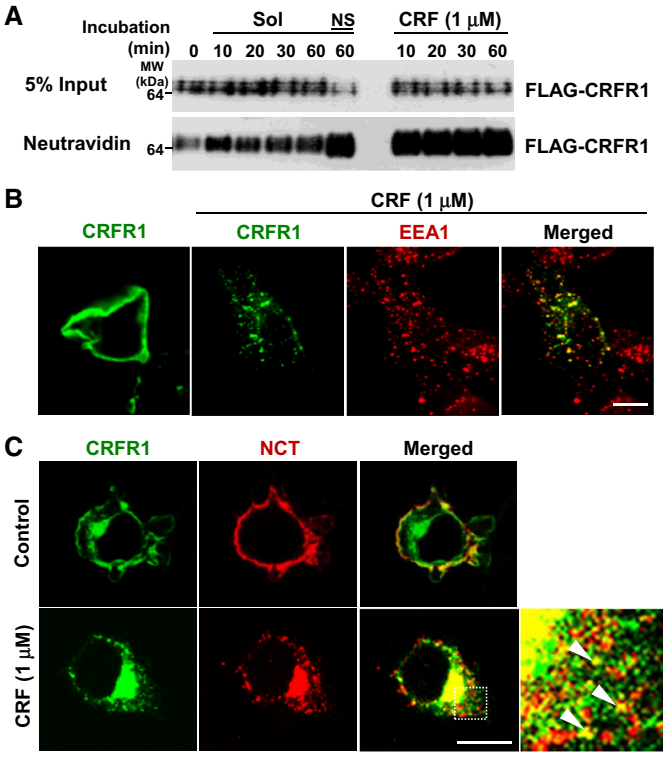

Figure 2. CRF treatment induces the internalization of CRFR1 and γ-secretase.

A Surface biotinylation and internalization assays show that CRF treatment significantly increases CRFR1 internalization (bottom panel, precipitated by Neutravidin beads) in SH-SY5Y cells stably expressing CRFR1. NS, no stripping. Sol, solvent treatment.

B CRFR1 is localized on plasma membranes and internalized following 1 h of CRF treatment in HEK293 cells. Internalized CRFR1 partially co-localized with an early endosomal marker protein, EEA1. Scale bar, 10 μm. The experiments were repeated three times.

C NCT, a γ-secretase subunit, is internalized upon CRF treatment. Live N2a ANPP-CRFR1 cells were stained with NCT antibody, treated with CRF, and incubated at 37°C to induce endocytosis. Scale bar, 10 μm. The experiments were repeated twice. Arrowheads indicate co-localization of NCT and CRFR1.

Source data are available online for this figure.

compared to the co-immunoprecipitation observed with the wild-type and Δ412 and IC3 mutants. As the Δ386/IC3 and Δ386 mutations impair the ability of the CRFR1 to recruit and bind β-arrestin2 (Oakley *et al*, 2007), these studies suggest that the interaction between CRFR1 and γ-secretase may require β-arrestin. To define a role of β-arrestin in association of CRFR1 and γ-secretase, we performed co-IP assays in wild-type, β-arrestin2 knockout (KO), and β-arrestin1 and 2 KO mouse embryonic fibroblasts (MEF). In the condition of PS1 and CRFR1 overexpression, PS1 was co-immunoprecipitated with CRFR1 in β-arrestin2 KO MEF, but the interaction is significantly reduced compared to that in wild-type MEF (Fig 3D, graph, PS1 interaction to CRFR1 normalized to PS1 levels in input). The interaction in β-arrestin1 and 2 KO MEF is further reduced compared to that in β-arrestin2 KO MEF, indicating that not only β-arrestin2 but also β-arrestin1 can contribute to the association of CRFR1 and γ-secretase (Fig 3D).

CRF increases the distribution of γ-secretase to lipid rafts

Many GPCRs, γ-secretase, and Aβ generation are localized in lipid rafts (Lee *et al*, 1998; Wahrle *et al*, 2002; Foster *et al*, 2003; Nabi & Le, 2003; Wada *et al*, 2003; Chini & Parenti, 2004; Vetrivel *et al*, 2004). We analyzed the localization of CRFR1 and γ-secretase in lipid rafts from SH-SY5Y CRFR1 stable cell lines. Under basal conditions, CRFR1 and PS1 were detected in raft fractions and more dense fractions (Supplementary Fig S3A and B). CRF treatment altered the distribution of PS1 in cells expressing wild-type CRFR1, but in cells expressing the CRFR1 Δ386/IC3 mutant, the distribution of PS1 was unchanged. Distribution of flotillin-1, a marker for lipid rafts, was not affected (Fig 3E). In multiple clonal lines, stable overexpression of wild-type CRFR1 also resulted in an increase in the PS1 in the raft fractions (Supplementary Fig S3C and D).

CRF modulates γ-secretase activity and Aβ generation through CRFR1-dependent and CRFR1-independent mechanisms

The effects of the four CRFR1 mutants on CRF-mediated increase in Aβ generation were examined in H4 human neuroglioma cells overexpressing wild-type APP695 (H4-APP695wt cells). Only the CRFR1 Δ386/IC3 mutant failed to show the CRF-mediated increase in Aβ40 and total Aβ compared to cells overexpressing wild-type. However, both the wild-type and mutant CRFR1-expressing cells showed increased Aβ42 levels upon CRF treatment (Fig 3F).

To ascertain whether this increase in Aβ42 could also be attributable to direct modulation of γ-secretase activity by CRF, three different *in vitro* γ-secretase activity assays were performed. First, a broken cell assay derived from H4 cells using stably overexpressing substrate was utilized (Ran *et al*, 2014). Membrane fractions including γ-secretase and the APP C-terminal fragment, C99, were isolated from cells, and incubated with CRF. In this assay, both Aβ40 and Aβ42 production were significantly increased by 25 μM CRF (Fig 4A). Second, an *in vitro* time-course assay was performed using the exogenous recombinant APP C-terminal fragment, C100, as a substrate with H4 cell membranes suspended in 0.25% CHAPSO as the source of γ-secretase. The rate of Aβ production was significantly increased in the presence of 25 μM CRF (Fig 4B). Finally, a reconstituted *in vitro* γ-secretase assay was used (Osenkowski *et al*, 2008; Holmes *et al*, 2012). 20 μM CRF increased Aβ production, with a trend toward a selective increase in Aβ42 observed at 50 μM CRF (Fig 4C).

CRFR1 antagonists increase Aβ production and do not block stress-mediated increases in Aβ levels

Given evidence for both CRFR1-dependent and CRFR1-independent effects of CRF on γ-secretase and Aβ production, we explored whether CRFR1 antagonists could alter Aβ production. In cell culture studies, non-selective CRFR antagonists astressin or alpha-helical CRF 9–41 (α–h 9–41) (Fig 5A) increased Aβ levels at low μM concentrations. In addition, CRF-mediated increases in Aβ were not blocked by these compounds (Fig 5A). Two small molecule CRFR1-specific antagonists NBI-27914 and antalarmin selectively increased Aβ42 (Supplementary Fig S5, Fig 5C and D). Both peptide antagonists, which bind to the extracellular N-terminal

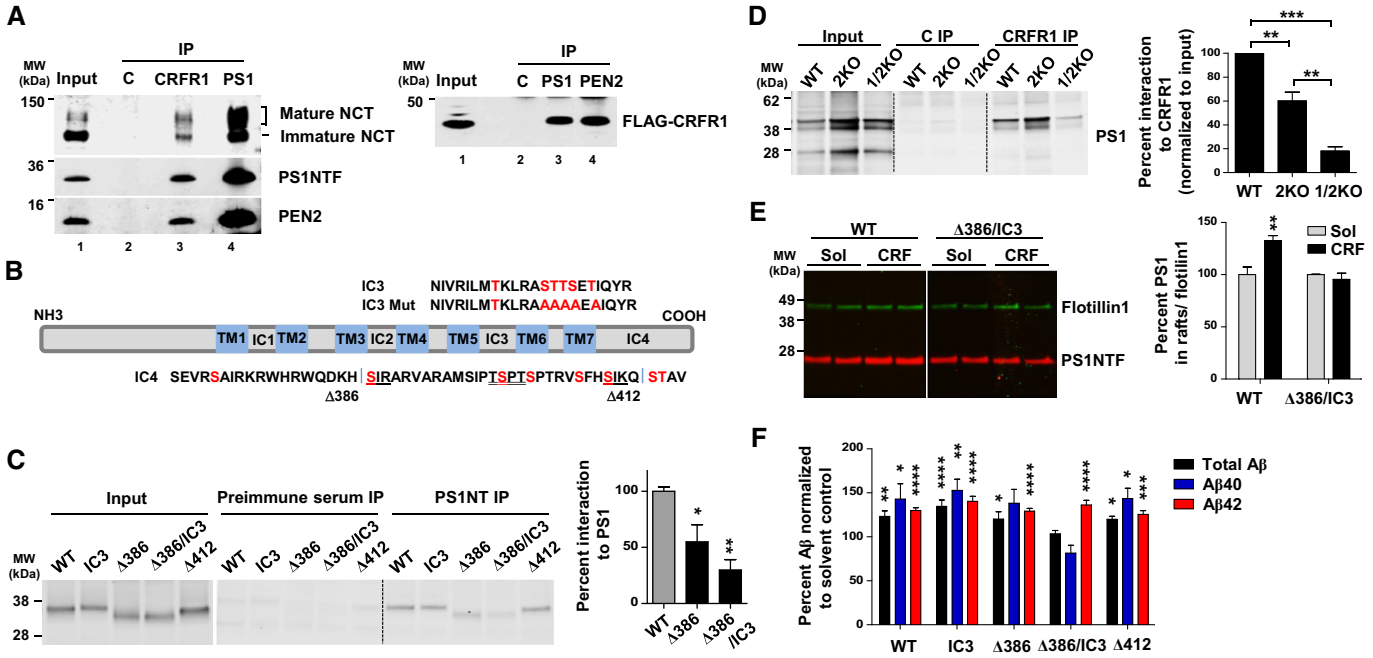

Figure 3. The C-terminal domain of CRFR1 mediates the effect of CRF on Aβ levels.

A CRFR1 associates with γ-secretase complex components. NCT, PS1, and PEN2 are co-immunoprecipitated with CRFR1 in N2a ANPP-CRFR1 cells (left panel). CRFR1 is co-immunoprecipitated with PS1 or PEN2 in N2a ANPP-CRFR1 cells. C, negative control with an irrelevant antibody (right panel).

B Schematic diagram of CRFR1 and sequences of intracellular 3 (IC3) and 4 (IC4). Highlighted in red, potential sites for phosphorylation; underlined, potential sites for protein kinase C (PKC) phosphorylation; double underlined, potential arrestin binding site.

C The CRFR1 Δ386 and Δ386/IC3 mutants which have less binding affinity to arrestin significantly decreased interaction to PS1. CRFR1 is overexpressed in HEK293 cells and is co-immunoprecipitated with PS1. CRFR1 wild-type, Δ386, and Δ386/IC3 mutants were used for the repeated co-immunoprecipitation experiments (n = 5/wild-type, n = 4/mutant).

D CRFR1 association with γ-secretase requires β-arrestins. Association of PS1 and CRFR1 is markedly reduced in β-arrestin1 and 2 knockout (KO) mouse embryonic fibroblast (MEF), compared to those in wild-type and β-arrestin2 KO MEF (n = 3). C IP, negative control with an irrelevant antibody.

E PS1 levels in lipid rafts are significantly increased by 1-h treatment of 1 μM CRF in CRFR1-expressing H4 APPwt cells. However, the CRFR1 Δ386/IC3 mutant did not mediate the effect of CRF treatment on PS1 levels in lipid rafts (n = 5/wild-type, n = 4/mutant).

F 1 μM CRF treatment into wild-type CRFR1-overexpressing H4 APPwt cells increased Aβ40, Aβ42, and total Aβ. However, CRFR1 Δ386/IC3 mutant-expressing H4 APPwt cells increased only Aβ42 by CRF treatment (n = 8).

Data information: Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 by one-way ANOVA with Tukey post-test (C, D) or two-way ANOVA and Bonferroni post-test (E, F). The experiments were repeated three times (C–F).
Source data are available online for this figure.

domain (N-domain), and CRFR1-selective non-peptide antagonists, which bind to the helical juxtamembrane domain (J-domain) centrally located within TM3 and TM5 of CRFR1 (Hoare *et al*, 2004), induced rapid internalization of CRFR1-GFP in HEK 293 cells at 5 μM (Supplementary Fig S4) consistent with a previous report (Perry *et al*, 2005). To test the functionality of the CRFR antagonists in our system, we measured the levels of cAMP produced from CRFR1 activation by CRF. Upon 15 min of CRF treatment, cAMP production was stimulated approximately 6-fold over basal levels. Both peptide and small molecule antagonists significantly blocked CRF-induced cAMP production (Supplementary Fig S6). The antagonists also increase Aβ production even though they significantly decrease cAMP levels, suggesting that the Aβ production induced by CRFR antagonists is not dependent on the intracellular cAMP signaling activation. To examine the effects of antagonist treatment on stress-induced Aβ increases, antalarmin was administered 30 min prior to restraint stress to C57BL/6J mice (Fig 5B). Consistent with our result in Fig 1A, vehicle-treated restraint stress mice displayed significantly more Aβ40 levels and an increasing trend of Aβ42 levels. However, the CRFR1 antagonist pre-stress injection did not block the effect of stress on Aβ levels.

The potential Aβ modulatory effects of CRF receptor antagonists were further elucidated using C99 stable H4 lines and analyzed with IP/MS (Fig 5D and Supplementary Fig S7). The CRFR1-selective non-peptide antagonists antalarmin and NBI-27914 significantly increased Aβ40 and Aβ42 levels, while they decreased smaller Aβ species: Aβs: Aβ37, Aβ38 and Aβ39 (Fig 5D). Antagonists were then tested for effects on Aβ production using *in vitro* γ-secretase assays. Astressin (5 μM) induced a modest increase (P = 0.06), whereas antalarmin and NBI-27914 had significant but complex effects on Aβ (Fig 5E). Notably, the selective increase in Aβ42 observed with antalarmin at 5 μM is reminiscent of the effects observed with many inverse γ-secretase modulators (iGSMs), though the decrease in Aβ40 and total Aβ observed at higher concentrations of both antalarmin and NBI-27914 is only observed with a subset of iGSMs (Golde *et al*, 2013).

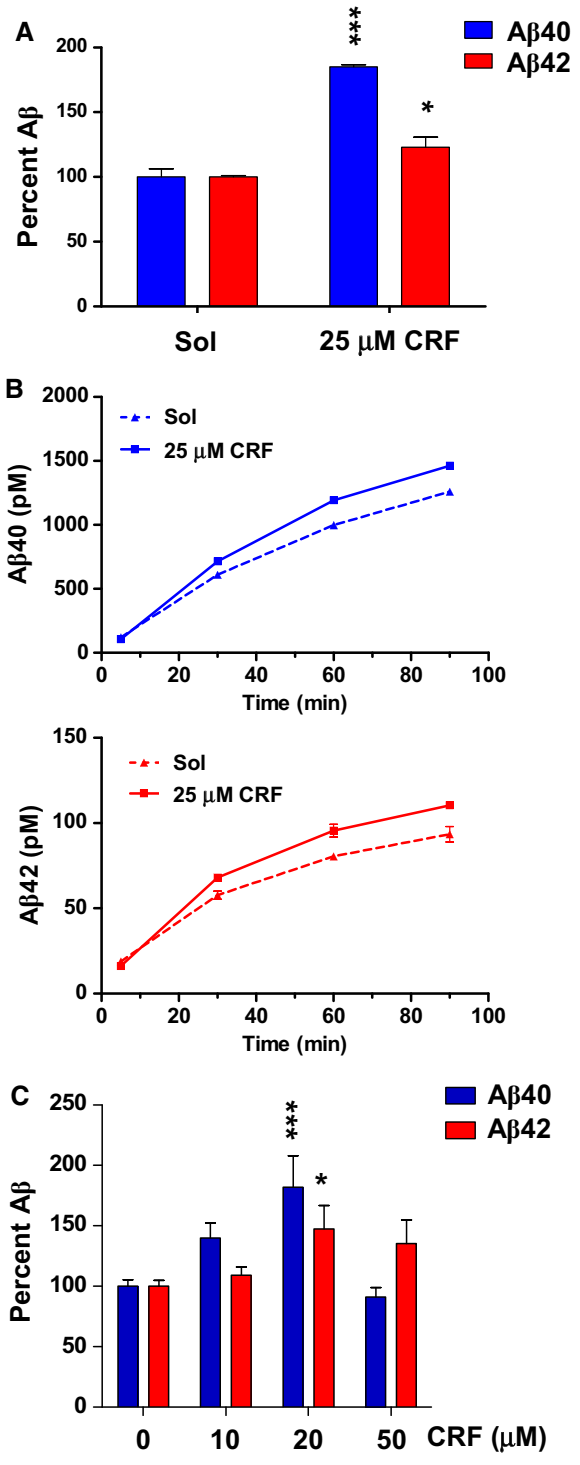

Figure 4. CRF increases γ-secretase activity *in vitro*.

A–C CRF increased Aβ40 and Aβ42 generation (A) in an *in vitro* γ-secretase activity assay using carbonate-extracted membranes from H4 cells stably expressing BRI-C99 (n = 3), (B) in an *in vitro* γ-secretase activity assay using exogenous substrate C100-FLAG (n = 2), and (C) in a reconstituted γ-secretase activity (n = 12 for vehicle alone, n = 9 for 10 μM, n = 6 for 20 and 50 μM). Data are presented as means ± SEM. *P < 0.05, ***P < 0.005, by unpaired t-test (A) or by two-way ANOVA and Bonferroni post-test (C). The experiments were repeated twice (A) or four times (C).

Discussion

Our studies demonstrate that CRF can increase Aβ production through (i) CRFR1-dependent alterations of γ-secretase localization into lipid rafts and (ii) direct actions on γ-secretase (Fig 6). Thus, there are two possible CRF-dependent mechanisms to explain how stress can increase Aβ levels in the brain (Kang *et al*, 2007; Carroll *et al*, 2011). Such data provide (i) a plausible biological basis for the observations that chronic stress in mice dramatically exacerbates Aβ pathology and (ii) clues as to how stress may contribute to AD risk. In some studies, preferential effects of CRF on Aβ42 are observed; an effect reminiscent of effects of fAD-linked *APP* and *PSEN* mutations that increase the relative level of Aβ42 (Borchelt *et al*, 1997). However, this phenomenon is not observed in other experiments reported here. Additional studies will be needed to establish whether other factors can mediate CRF's preferential action on Aβ42, as a combined effect on increasing total Aβ levels combined with an increase in Aβ42:Aβ40 ratios would be predicted to be more pathogenic than a general increase in total Aβ alone (Kim *et al*, 2007; Golde *et al*, 2013).

The CRF-CRFR1-mediated increases in Aβ can be attributed to association of CRFR1 with γ-secretase resulting in the internalization and redistribution of the CRFR1 γ-secretase complex to lipid rafts. This effect appears to require β-arrestin recruitment and binding as the CRFR1 Δ386/IC3 mutant (Oakley *et al*, 2007) that has been previously shown to decrease translocation of β-arrestin2 to CRFR1 (i) exhibited a reduced interaction with γ-secretase, (ii) blocked CRF-mediated redistribution of γ-secretase into rafts, and (iii) blocked the CRF-induced increase in total Aβ and Aβ40 but not Aβ42. Although we cannot exclude a role for β-arrestin1 in these studies (Fig 3D), β-arrestin2 has been shown to translocate more rapidly and strongly than β-arrestin1 to agonist-activated CRFR1 (Oakley *et al*, 2007). These studies complement other recent studies showing that GPR3 and the β2-adrenergic receptor regulate γ-secretase activity and Aβ through their recruitment and binding of β-arrestin2 (Ni *et al*, 2006; Thathiah *et al*, 2013). Nevertheless, both β-arrestins may have contributed to the formation and internalization of the CRFR1–γ-secretase complex considering that after the early rapid recruitment and binding of β-arrestin2 had occurred, significant β-arrestin1 would have then translocated to cell surface receptors during the 1-h CRF treatment (Hauger *et al*, 2009).

In contrast to the receptor-mediated effects of CRF, the direct stimulatory action of CRF on γ-secretase observed *in vitro* was highly unexpected. To our knowledge, CRF is the first endogenous neuropeptide with a positive modulatory effect on γ-secretase cleavage. We postulate that CRF acts as a positive allosteric modulator of γ-secretase activity. It is challenging to determine whether the receptor-dependent or receptor-independent effects of CRF account for the *in vivo* effects of acute stress on increasing γ-secretase. Our finding that non-peptide CRFR1 antagonists can act as inverse γ-secretase modulators and mediate internalization of CRFR1 thereby failing to block CRF-stimulated increases in Aβ formation indicates that these pharmacologic tools cannot be used to cleanly dissect the mechanism of action *in vivo*. A very recent study on subacute and chronic isolation stress in the APP Tg 2576 line, indicated that under these conditions and in this model 20 mg/kg antalarmin suppressed the stress-induced increase in Aβ levels (subacute stress) and Aβ deposition (chronic stress) (Dong *et al*,

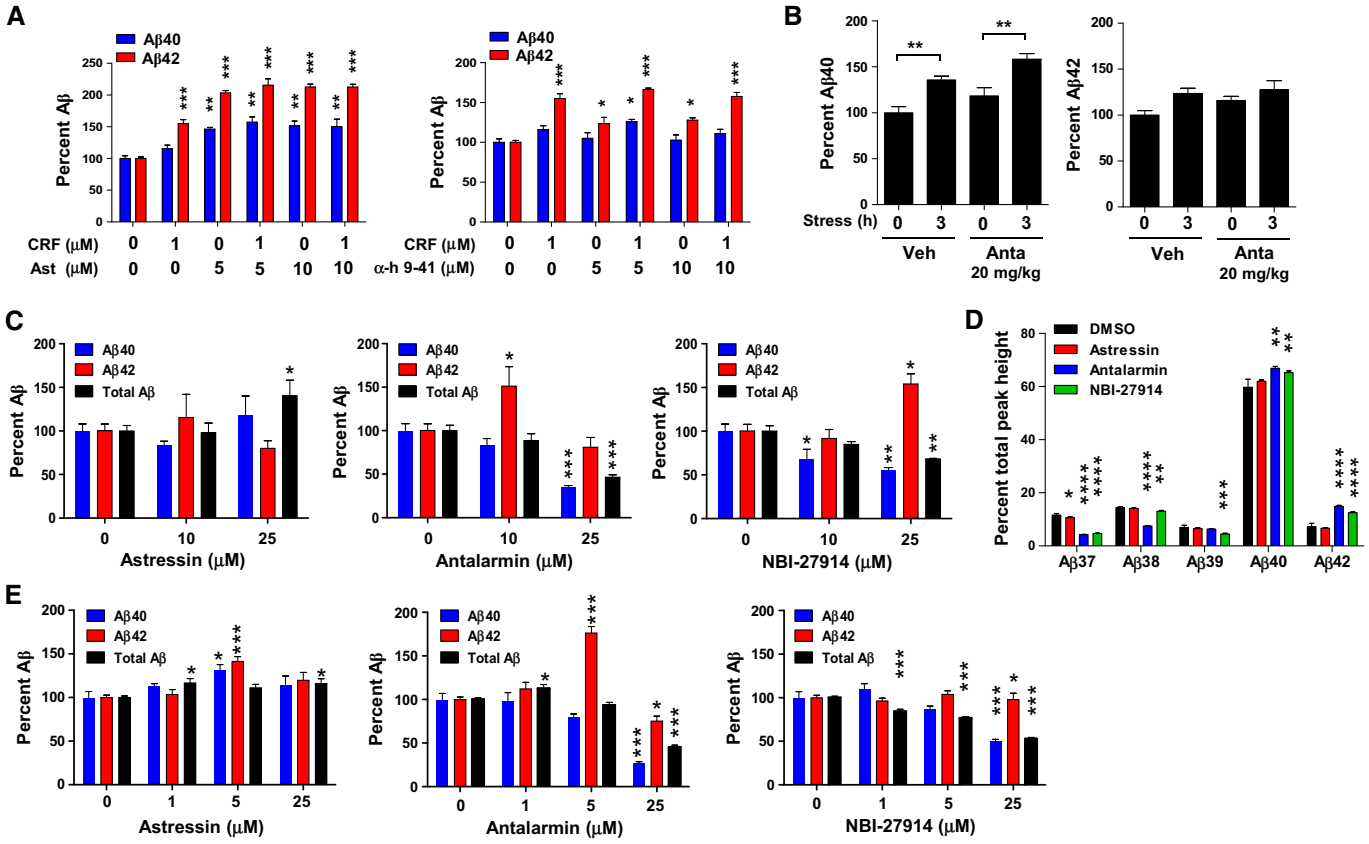

Figure 5. CRFR antagonists do not block CRF- and stress-induced Aβ increases and alter γ-secretase processivity.

A Peptidic CRFR antagonists, astressin (Ast) or α-helical CRF 9–41 (α–h 9–41) increased Aβ levels in SH-SY5Y cells and did not block CRF effects on Aβ generation (n = 3).

B Antalarmin treatment did not block stress-induced endogenous Aβ increases in C57BL/6J mice (n = 8/vehicle and antalarmin no stress; vehicle stress for Aβ42, n = 7/vehicle stress for Aβ40; antalarmin stress for Aβ42, n = 6/antalarmin stress for Aβ40).

C Antalarmin and NBI-27914 significantly increase the ratio of secreted Aβ42:Aβ40 in H4 cells stably expressing BRI-C99 (n = 8/vehicle for Aβ40 and Aβ42, n = 7/vehicle for total Aβ; antalarmin for Aβ42; n = 4/other group).

D IP/MS analysis showed that 10 μM antalarmin and NBI-27914 significantly decreased the production of short Aβ and increased longer Aβ in H4 cells stably expressing BRI2-C99 (n = 3).

E Aβ ELISAs of *in vitro* γ-secretase activity assay using exogenous substrate C100-FLAG further proved the iGSM-like activity of Antalarmin and NBI-27914 (n = 5/vehicle for Aβ40 and Aβ42, n = 6/other group).

Data information: Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 by one-way ANOVA and Tukey post-test. The experiments were repeated twice (A, C and E).

2014). These data are difficult, at present, to reconcile with our results showing that 20 mg/kg antalarmin did not block the rise in brain Aβ upon acute stress. However, the discrepant results could reflect differences in acute versus subacute and chronic dosing paradigms or alternatively the use of transgenic mice as opposed to non-transgenics. In addition, the Dong *et al* study used relatively small group sizes of transgenic mice for both subacute and acute studies. Furthermore, they only reported the levels of the PBS-solubilized Aβ fraction, which in that line of mice represents ~5% or less of total brain Aβ and in mice with amyloid deposits does not accurately reflect actual amyloid loads (Kawarabayashi *et al*, 2001). In addition to CRF and CRFR1, the CRFR cell signaling system includes three other CRFR ligands, urocortin (UCN) 1, 2, and 3 (Reul & Holsboer, 2002; Hauger *et al*, 2006). Considering the exquisite complexity of the CRF system—especially the high affinity of UCN1 for CRFR1 and the specialized regional expression of CRF and UCNs in brain neurons—examination of possible UCN1 roles in γ-secretase modulation will be important.

Understanding the biological basis of how non-genetic risk factors may contribute to AD pathogenesis is challenging. Risk factors may be temporally uncoupled from a subsequent diagnosis of AD by decades; thus, epidemiologic studies are subject to concerns regarding potential confounds. Indeed, validation of a non-genetic risk factor implicated from epidemiological studies requires that repeated evidence for risk be coupled with compelling biology demonstrating the risk factor can mechanistically alter brain physiology in a way that could contribute to AD pathogenesis. Previous studies have provided plausible links between stress, CRF, CRFR1, and tau pathology mediated by CRF:CRFR1-dependent stress-induced activation of tau kinases (Rissman *et al*, 2007, 2012;

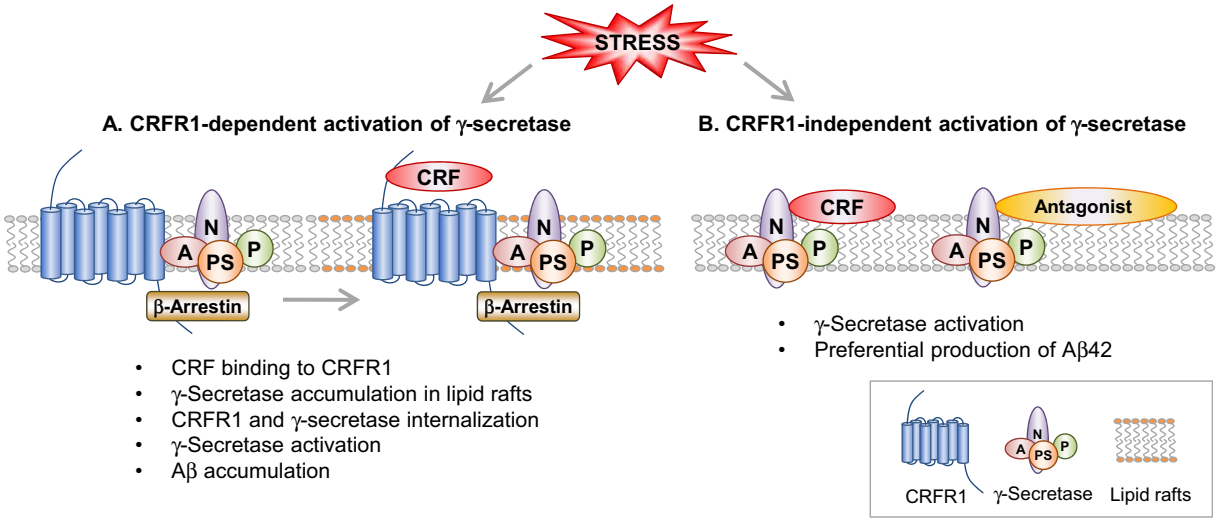

Figure 6. Models for the role of CRF and CRFR antagonists in the localization and activation of γ-secretase and Aβ production in Alzheimer's disease.

A γ-secretase interacts with CRFR1 through β-arrestins. Upon CRF binding to CRFR1, γ-secretase–CRFR1 complex moves into lipid rafts and the complex is internalized to endosomes, where γ-secretase activity increases. CRFR antagonists also induce internalization of CRFR1, suggesting another possible mechanism that the antagonists activate γ-secretase through CRFR1 (Supplementary Fig S4).

B Both CRF and CRFR antagonists activate γ-secretase *in vitro*. Aβ42 productions preferentially increase by CRFR1-independent γ-secretase activation by CRF and the antagonists.

Carroll *et al*, 2011). Here, we provide mechanistic insight into the previous observation (Kang *et al*, 2007; Carroll *et al*, 2011) that CRF could increase Aβ production *in vivo* and accelerate amyloid pathology in APP mouse models. Collectively, these data provide converging biological data that stress response meditated by CRF:CRFR1 could contribute to AD pathogenesis. Antagonism of this pathway has been proposed as a potential therapeutic approach to AD, but our data showing that CRFR1 antagonism does not achieve the desired effect on acute stress-induced Aβ production and under some circumstances can directly augment Aβ production with a preferential effect on Aβ42 suggests that use of CRFR1 antagonists with these properties may promote rather than suppress amyloid pathology. Instead, our data would suggest (i) that direct targeting of CRF perhaps via an anti-CRF antibody approach or (ii) a G protein-biased CRFR1 agonist that does not result in β-arrestin recruitment to CRFR1 might be necessary to effectively target this pathway for therapeutic benefit in AD.

Materials and Methods

Restraint stress

Thirteen- to 14-week-old male and female C57BL/6J mice (Jackson Laboratory) were utilized. For restraint, each mouse was placed in a ventilated 50-ml conical tube (Falcon) for 3 h. Mice were not physically squeezed and experienced no pain. They could rotate from a supine to prone position, but not turn head to tail. Non-restrained mice remained in their home cages in the experimental room. Mice were randomly assigned to experimental groups and were housed in a constant 12-h light/dark cycle with free access to laboratory rodent chow at all times. All procedures are approved by the University of Florida IACUC. All tissue samples from *in vivo* experiments were randomly renumbered, and the investigators were blinded during sample analysis to avoid subjective bias. A pilot study with 6–8 animals was performed and the samples size was adjusted when experiments were repeated.

Primary culture from mouse brain

Cortices were isolated from neonate wild-type C57BL/6J mice. Tissues were dissociated with papain solution (Worthington) and 50 μg/ml DNase I (Sigma) at 37°C for 20 min. After digestion, cortices were washed three times with Hank's balanced salt solution (GIBCO) to remove the papain and placed in media consisting of Neurobasal (Life Technologies) supplemented with 0.02% Neurocult SM1 (Stemcell), 0.5 mM Glutamax, 5% Fetal Bovine Serum (Hyclone) and 0.01% Antimycotic–Antibiotic (GIBCO). The tissue was triturated in the same media and dissociated cells were plated in a 24-well Poly-D-lysine (Sigma)-coated plate at a density of 200,000 cells per well as described (Sacino *et al*, 2013). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

Cell culture and transfection

SH-SY5Y (American Type Culture Collection, ATCC) and HEK293 cells (Park *et al*, 2012), and mouse embryonic fibroblast (MEF) cells were cultured as recommended by ATCC. N2a-ANPP cells (Kim *et al*, 2003) were maintained in 200 μg/ml hygromycin B (Life Technologies) and 200 μg/ml G418 (Life Technologies). For transfections, Lipofectamine® 2000 (Life Technologies) was used according to manufacturer's instructions. To generate CRFR1 stable cell lines, SH-SY5Y cells were transfected with pAG3 Zeo FLAG-CRFR1 and N2a-ANPP cells (Kim *et al*, 2003) were transfected with pAG3 Zeo

FLAG-CRFR1 and pBLAST (InvivoGen). Cells were selected as required by using 200 μg/ml zeocin (Life Technologies) or 3 μg/ml blasticidin (Life Technologies). H4 stable cells expressing BRI2-C99 (Ran *et al*, 2014) were maintained in 200 μg/ml hygromycin B. All cell lines used for this study were tested for mycoplasma contamination.

DNA constructs and antibodies

CRFR1 cDNA (GenBank ACC# AY457172) was from the University of Missouri S&T cDNA Resource Center. The cDNA was tagged with turboGFP at the C-terminus to generate CRFR1-GFP construct. To generate the FLAG-CRFR1 construct, the signal peptide of CRFR1 was substituted with the hemagglutinin signal sequence (MKTIIAL SYIFCLVFA) (Jou *et al*, 1980) and FLAG epitope (DYKDDDDK) (Hoare *et al*, 2005; Perry *et al*, 2005). For co-immunoprecipitation experiments with mutant CRFR1 (Fig 3), cDNAs were previously synthesized for HA-tagged CRFR1 with truncated C-termini (Δ412, Δ386) and mutated STTSET motif in the third intracellular loop (Oakley *et al*, 2007).

Antibodies used in this study are summarized in Table 1.

Drug preparation and treatment

Human/rat CRF (H-2435), astressin (H-3422), and α-helix CRF9–41 (H-2040) were purchased from Bachem (King of Prussia), and a stock solution was prepared in 10 mM acetic acid. Corticosterone (27840), antalarmin (A8727), and NBI-27914 (N3911) were purchased from Sigma, and stock solutions were prepared in DMSO. For *in vivo* treatment, antalarmin was prepared in Solutol® HS 15 (BASF)/ethanol/water at a ratio of 15:10:75 including up to 4.5% DMSO. Antalarmin was administered at 20 mg/kg by intraperitoneal injection 30 min before restraint stress. 1 μM CRF was used for all the experiments except for primary culture (10 μM) and *in vitro* experiments. Cells were treated with CRF or antagonists for 12–16 h unless indicated differently in the figure legends.

Table 1. Summary of antibody epitopes.

Antibody	Target protein (source)
A8717	Amino acid 676–695 of APP695 (Sigma, A8717)
PS1NT	Amino acid 1–65 of PS1 (Kim & Sisodia, 2005a,b)
β-actin	β-actin (Sigma, A1978)
FLAG M2	DYKDDDDK (Sigma, F1804)
EEA1	EEA1 (BD Biosciences, 610465)
NCT54	Amino acids 242–546 of NCT (Kim *et al*, 2003)
CT11	Last 7 amino acids of APLP1 (von Koch *et al*, 1997)
HA	YPYDVPDYA (Roche, clone 3F10; Sigma, clone HA-7)
Flotillin-1	Flotillin-1 (BD Biosciences, 610821)
P2-1	Ectodomain of APP (Van Nostrand *et al*, 1989)
82E1	N-terminal-end of Aβ (IBL, 10323)
PNT2	Amino acids 1–26 of PEN2 (Vetrivel *et al*, 2004)
KDEL	GRP94 and GRP78 (Stressgen, clone 10C3)
13.1.1	Aβ35–40 (Levites *et al*, 2006a)
2.1.3	Aβ35–42 (Levites *et al*, 2006a)
AB5 HRP-AB5	Human Aβ1–16 (Levites *et al*, 2006a) horseradish peroxidase conjugated AB5
Signet9153	Aβ1-x (Lanz & Schachter, 2006, 2008; Yohrling *et al*, 2007)
R162	Aβx-40 (Lanz & Schachter, 2006, 2008; Yohrling *et al*, 2007)
R164	Aβx-42 (Lanz & Schachter, 2006, 2008; Yohrling *et al*, 2007)
Aβ38	Aβ38 (Lanz & Schachter, 2006, 2008; Yohrling *et al*, 2007)
HRP-4G8	Aβ17–24 horseradish peroxidase conjugated (Covance)

Aβ ELISA

Human Aβ ELISA using conditioned cell culture medium and rodent Aβ ELISA using mouse forebrain homogenates were performed as described previously (Lanz & Schachter, 2006, 2008; Levites *et al*, 2006b; Yohrling *et al*, 2007; Park *et al*, 2012). Immulon 4HBX flat bottom 96-well plates (Thermo Scientific) were coated with either monoclonal antibodies specific to Aβ40 (13.1.1, human Aβ35–40 specific), Aβ42 (2.1.3, human Aβ35–42 specific), or total Aβ (AB5, human Aβ1–16 specific), and blocked with 1% BSA. Conditioned cell culture medium or Aβ peptides standard were added. Horseradish peroxidase (HRP)-conjugated 4G8 (Covance) or AB5 antibodies were used as detection antibodies. Tetramethylbenzidine substrate was added, and then 6.7% phosphoric acid was added to stop the reaction. Absorbance values were read at a wavelength of 450 nm. The signal generated by the CRF peptide alone was no higher than the background signal from buffer alone, indicating that there is no cross-reactivity of the CRF peptide to the Aβ ELISA (Supplementary Fig S8). For rodent Aβ ELISAs, plates were coated with end-specific monoclonal antibodies to Aβ1-x, Aβx-40, Aβx-42, or Aβ38 respectively. After adding the cell culture medium or tissue lysates, Aβ was detected with HRP-conjugated 4G8 and exposed with QuantaBlu Fluorogenic Peroxidase Substrate (Pierce Chemical). Mouse forebrains were homogenized in 0.4% Diethylamine/Sodium Chloride (DEA/NaCl) extraction buffer and Aβ recovered and purified as described on 150 mg HLB Oasis columns (Waters), as described.

Western blotting and sample preparation

Cell lysis and Western blotting were performed as described previously (Kim *et al*, 2004; Park *et al*, 2012). Detergent lysates of cells were prepared using immunoprecipitation buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) supplemented with a protease inhibitor cocktail (P8340; Sigma). Lysates were subjected to SDS–PAGE and transferred to nitrocellulose membranes prior to incubation with selected antibodies. Immunoblots were developed using either the enhanced chemiluminescence (ECL) detection system (PerkinElmer) or an Odyssey infrared scanner (LiCor Biosciences).

Live cell staining and laser confocal immunofluorescence microscopy

Cells grown were transfected with CRFR1-GFP for 24 h and incubated with an affinity-purified NCT54 antibody at 10°C for 30 min without fixation or detergent permeabilization. After cells were washed with serum free medium, cells were treated with CRF or CRFR antagonists at 37°C for 30 min. Cells were fixed in 4%

paraformaldehyde in PBS for 10 min, permeabilized in 0.2% Triton X-100 in PBS for 10 min, and incubated with Alexa594-labeled anti-rabbit secondary antibody (Life Technologies) for 1 h. Following washing and mounting onto glass slides images were obtained with a TCS SP2 AOBS Spectral Confocal Microscope (Leica) or Olympus DSU-IX81 Spinning Disc Confocal Microscope (Olympus).

Receptor internalization assay (modified surface biotinylation)

Cells were incubated in 0.5 mg/ml sulfosuccinimidobiotin (Pierce) at 4°C for 20 min. After cells were washed with PBS-CM (PBS with 1 mM $CaCl_2$ and $MgCl_2$), cells were treated with CRF or CRFR antagonists at 37°C for 30 min. To remove surface biotin, cells were incubated in reducing agent glutathione (50 mM glutathione, 75 mM NaCl and 10 mM EDTA, pH 8.75) for 30 min on ice, and glutathione was then neutralized with 27 mM iodoacetamide in PBS-CM for 10 min on ice. The cells were lysed with immunoprecipitation buffer containing protease inhibitors, adjusted to 0.25% SDS, boiled for 10 min, and incubated with 50 μl of streptavidin-agarose beads (Pierce) at 4°C overnight. The captured proteins and 5% of the lysates used for precipitation were resolved on 6% tris-glycine gels, blotted, and probed with NCT54 antibody.

Co-immunoprecipitation

Cells were washed twice with ice-cold PBS and solubilized in CHAPS Co-IP buffer (1% CHAPS (Calbiochem), 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA). Lysates were centrifuged at 16,100 × *g* for 10 min at 4°C, and the resulting supernatant was used for co-immunoprecipitation with respective antibodies at 4°C overnight. Immune complexes were collected with Protein A- or G-conjugated agarose beads (Pierce) and eluted in SDS sample buffer.

Lipid raft isolation

Lipid raft isolations were performed as described previously (Wahrle *et al*, 2002). Cells were pelleted and then lysed in 2% CHAPSO (Calbiochem) and 0.15 M Na Citrate (pH 7.0) with complete PI. After incubation on ice for 15 min, the lysates were spun at 1,000 *g* for 10 min at 4°C. The cleared lysate was then sequentially diluted with sucrose containing 0.15 M sodium citrate (pH 7.0) so that the final concentration of CHAPSO was 0.25% and sucrose was 45%. Four milliliters of this homogenate was then applied to the bottom of the centrifuge tube, and sequentially overlaid with 4 ml of 0.15 M sodium citrate (pH 7.0), 35% sucrose, 0.25% CHAPSO followed by 4 ml of 0.15 M sodium citrate (pH 7.0), 5% sucrose, 0.25% CHAPSO. The tubes were subjected to ultracentrifugation at 260,000 *g* for 18 h in Beckman SW41 rotor at 4°C. Twelve 1-ml fractions were collected from the top of the gradient using a fractionator, and 60 μl of each fraction was analyzed by Western blotting.

In vitro γ-secretase activity assays

Cell-free γ-secretase activity assays were performed as described previously (McLendon *et al*, 2000; Fraering *et al*, 2004), from carbonate-extracted membranes derived from H4 cells expressing BRI2-C99. For the *in vitro* assay with exogenous substrate, C100-FLAG was purified as described previously (Ran *et al*, 2014). Carbonate-extracted membrane was derived from CHO cells and diluted to final total protein concentration at 2 mg/ml. 1 μM C100-FLAG was incubated with 10 μl membranes in total volume of 200 μl sodium citrate buffer [150 mM, pH 6.8, 0.3% CHAPSO (Calbiochem), 1× complete protease inhibitors (#11697498001, Roche)]. For the time course experiment, an aliquot of 30 μl was taken out every 30 min and left on ice with 2 μM of the GSI LY-411,575. The other tests with antagonists were incubated for 90 min. Reconstituted γ-secretase activity assays were performed as described previously (Holmes *et al*, 2012). Immunoprecipitation and mass spectrometry of Aβ production in cell culture media and *in vitro* assay were performed as described previously (Ran *et al*, 2014). The γ-secretase activity assays used in this study are summarized in Supplementary Table S1.

RNA extraction and qRT–PCR

RNA was extracted using PureLink RNA mini kit (Life Technologies), following the manufacturer's instructions, and the concentrations were determined using NanoDrop spectrophotometer (NanoDrop Technologies). RNA was converted into cDNA using Superscript III First-Strand synthesis supermix (Life Technologies). All reverse transcriptase steps followed the protocols of the respective manufacturer. The qRT–PCRs were performed using SsoFast Probes Supermix (BioRad) with 0.3 nM of CRFR1 primers (Schoeffter *et al*, 1999).

Measurement of cAMP

After washing with Opti-MEM (Life Technologies) including 0.5 mg/ml vitamin C (Sigma) and 1 mg/ml BSA (Sigma) twice, SHSY5Y CRFR1 cells were treated with 5 μM of astressin or 10 μM of antalarmin for 1 h and then treated with 1 μM CRF for 15 min in Opti-MEM including 1 mM 3-isobutyl-1-methylxanthine (Sigma), 0.5 mg/ml vitamin C (Sigma), and 1 mg/ml BSA. The intracellular cAMP content was determined from the cell lysates using the cAMP paprameter assay kit according to manufacturer's instruction (R&D systems).

Statistical analysis

Data were graphed as means ± SEM using Prism 5 software (GraphPad). analysis was as described using Student's *t*-test, one-, or two-way ANOVA.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

HJP, SHK, TEG, and KMF designed research and analyzed data; HJP performed most of the research; YR, JIJ, and OH performed *in vitro* γ-secretase activity assay; RLH provided CRFR1 mutant constructs and intellectual input on CRFR1, contributed cAMP assay, and edited the manuscript; ARP and LS contributed to acute stress induction experiments; CC prepared mouse brain primary cultures; CH contributed to establishment of stable cell lines; MSW analyzed *in vitro* γ-secretase activity assay data and edited the paper; YD provided β-arrestin2 and β-arrestin1/2 knockout MEF; AER provided intellectual input and edited the manuscript; HJP, KMF, and TEG wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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