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BRAIN
REPORT

Sex-dependent effects of acute stress on amyloid-β in male and female mice

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The risk of developing Alzheimer's disease is mediated by a combination of genetics and environmental factors, such as stress, sleep abnormalities and traumatic brain injury. Women are at a higher risk of developing Alzheimer's disease than men, even when controlling for differences in lifespan. Women are also more likely to report high levels of stress than men. Sex differences in response to stress may play a role in the increased risk of Alzheimer's disease in women.

In this study, we use *in vivo* microdialysis to measure levels of Aβ in response to acute stress in male and female mice. We show that Aβ levels are altered differently between female and male mice (APP/PS1 and wild-type) in response to stress, with females showing significantly increased levels of Aβ while most males do not show a significant change. This response is mediated through β-arrestin involvement in Corticotrophin Releasing Factor receptor signalling pathway differences in male and female mice as male mice lacking β-arrestin show increase in Aβ in response to stress similar to females.

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Introduction

Chronic stress causes damage or dysfunction in the hippocampus, resulting in impaired memory in animal models[1,2] and humans.[3,4] Animal studies have shown that stress increases both Aβ and tau pathology[5,6] in the brain. Demonstrating the direct impact of stress in humans has proven difficult, as stress is a factor in numerous complex psychological conditions. Patients scoring high for 'distress proneness' are 2.7-fold more likely to be diagnosed with dementia in the next 3 years,[7] and individuals who experience late-life depression have a 2-fold elevated risk of a dementia diagnosis.[8] In fairness, some studies have not found a correlation between stress/depression symptoms and Alzheimer's disease,[9] highlighting the complexity of the topic and need to use experimental models to dissect the biology underlying this linkage.

Results from both human and animal studies have identified a clear increase in the incidence of Alzheimer's disease in females, regardless of the increased lifespan in women.[10] Sex differences in the prevalence or severity of many diseases are well recognized. Of the disorders that are more prevalent in females, a common underlying feature is an association with stress.[11,12] Stress

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increases glucocorticoids that have been associated to Alzheimer's disease.[13,14] In patients, elevated glucocorticoid levels correlated with dementia severity.[15]

Corticotropin Releasing Factor (CRF) initiates the hypothalmic-pituitary-adrenal axis response to stress in the body and signals through receptors (CRF-R) in the brain. Brains of patients with Alzheimer's disease have increased CRF-R1 density[16] compared to age-matched controls, and overexpression of CRF increases Aβ pathology in APP transgenic mice.[17] Sex biases in CRF-R receptor trafficking were shown in locus coeruleus neurons in rats.[18] During stress in females, CRF-Rs activate a protein kinase A (PKA) and extracellular regulated kinase (ERK) cascade[19] to, among other things, increase excitability of those neurons.[20] In males, however, β-arrestin removes CRF-R from the cell surface during stress, thus significantly reducing CRF-R signalling.[17,18] It is possible that sex-related differences in CRF-R signalling or trafficking could underlie differences in molecular and cellular responses to stress in different areas of the brain as well.

In 2007, our laboratory demonstrated that 3 h of restraint stress (RS) resulted in an increase in Aβ in hippocampal interstitial fluid (ISF).[21] While both male and female mice were included in that study, we did not assess a potential sex difference. The purpose of this study is to determine whether a sex difference exists in that response and identify possible mechanisms accounting for the difference.

Materials and methods

Animals

All experimental procedures were performed in accordance with guidelines established by the Institutional Animal Care and Use Committee at Washington University and following ARRIVE guidelines. Animals were housed in standard cages with nestlets in a room with 12/12 light/dark cycle. We bred APP/PS1$^{+/-}$ hemizygous mice (RRID:MMRRC 034829-MU; Jackson Laboratories, Bar Harbor, ME, USA) to wild-type C3H/B6 mice (RRID:IMSR JAX:100010). β-arrestin1 KO mice (RRID:IMSR JAX:011131) were obtained and bred to establish a colony. Male and female littermate mice were evaluated separately, and littermates were equally distributed and randomly assigned to experimental groups. Group sizes are powered to detect a 20% change in Aβ based on our previous studies.[22] A cohort of control C57Bl6 mice (RRID:IMSR JAX:000664) were obtained for western blot and staining experiments.

Acute stress

In the RS condition, mice were placed in a 50 ml plastic conical with ventilation holes for 3 h, beginning at the onset of the dark cycle. In microdialysis experiments, ISF was sampled for up to 18 h following the cessation of stress. In the western blot experiments, animals were euthanized immediately following 3 h of stress. Predator olfactory stress: during microdialysis experiments, a microcentrifuge tube containing a Kimwipe with 0.1 ml of fox, coyote or bobcat urine (Wildlife Research Center, Ramsey, MN, USA) was placed into the home cage of each mouse. The tube was exchanged out every 30 min with a different scent to avoid habituation to the stressor. Total duration of olfactory stress was 3 h.

In vivo microdialysis

In vivo microdialysis to assess brain ISF Aβ in the hippocampus was performed as previously described.[22] Under isoflurane, guide cannula (BR-style, Bioanalytical Systems) were cemented above the left hippocampus. Mice recovered for 5–7 days on a 12–12 light/dark cycle to re-habituate after surgery, then 2 mm microdialysis probes were inserted into the hippocampus (BR-2, 30-kilodalton molecular weight cut-off membrane, Bioanalytical Systems). Perfusion buffer was artificial CSF containing 0.15% bovine serum albumin (Sigma) filtered through a 0.22 μM membrane. Flow rate was a constant 0.5 or 1.0 μl/min. Samples were collected every 60–180 min with a refrigerated fraction collector and assessed for $Aβ_{x\text{-}40}$ or $Aβ_{x\text{-}42}$ by enzyme-linked immunosorbent assay ELISA. Basal ISF Aβ for each mouse is calculated as the mean 9 h before stress or drug pretreatment. All timepoints are normalized to the basal ISF Aβ concentration of that mouse.

Compounds

Compounds were administered by reverse microdialysis diluted in artificial CSF containing 0.15% bovine serum albumin (Sigma) for perfusion through the microdialysis probe. Antalarmin (Sigma) was diluted to 4 nM before perfusion. KT5720 (Tocris), a selective PKA inhibitor was diluted to 6 mM before perfusion. FR180204 (ThermoFisher), an ERK inhibitor, was diluted to 100 mM.

ELISA

ISF Aβ concentrations were assessed using an in-house sandwich ELISAs as described.[22] Mouse anti-$Aβ_{40}$ (mHJ2) or anti-$Aβ_{42}$ m(HJ7.4) antibodies were used to capture, biotinylated mHJ5.1 for detection, followed by streptavidin-poly-HRP-40 (Fitzgerald Industries) and developed using Super Slow ELISA TMB (Sigma). Absorbance was read on a Bio-Tek Epoch plate reader at 650 nm. Human $Aβ_{1–40}$ or $Aβ_{1–42}$ peptides (Anaspec) were used as the standard. For corticosterone measurements, plasma samples were assessed using a Corticosterone ELISA kit (Cayman Chemical) following the standard protocol.

Statistical analysis

Data were analysed using statistical software (GraphPad Prism 9, San Diego, CA, USA). All data were screened for normality before statistical analysis. Data analysis was conducted blind to treatment groups and by different experimenters than those running microdialysis. One- and two-way ANOVAs and repeated measures ANOVAs were used to analyse group differences. The Geisser–Greenhouse correction was applied in repeated measures models to protect against violations of sphericity and compound symmetry. The Bonferroni correction was applied to multiple pairwise comparisons, and the two-stage linear step-up procedure was applied to interpret significant interactions while correcting for multiple comparisons by controlling the false discovery rate. Statistical significance was set at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. A statistical summary is provided in Supplementary Table 1.

Methods for cytosolic and cell membrane fractionation, western blotting and immunohistochemistry can be found in the Supplementary material.

Data availability

Data are available upon request.

Results

Aβ levels in the hippocampus following stress increase significantly more in female mice compared to male mice

In female mice, ISF $Aβ_{40}$ levels increased by ∼50% within 2 h of the onset of stress and remain elevated for at least 14 h (Fig. 1A). In

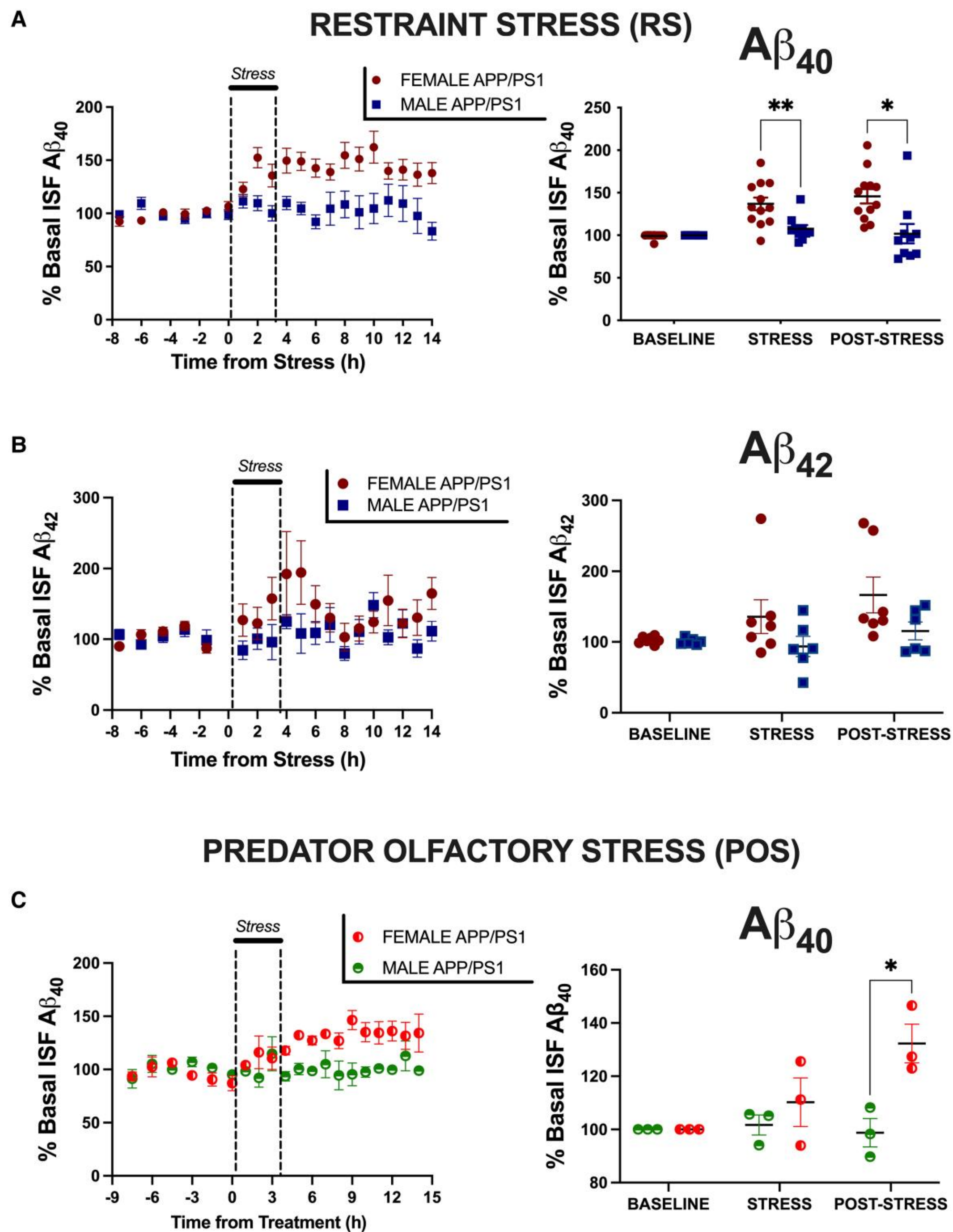

Figure 1 Sex-dependent increase in ISF Aβ during stress. (**A**) Using *in vivo* microdialysis, in females RS increased ISF $A\beta_{40}$ levels [two-way RM ANOVA main effect of Sex $F(1,20) = 16.12$, $P = 0.0007$] by $136.9 \pm 7.4\%$ (*post hoc* comparison, $P = 0.0098$) during stress and levels remained elevated at $145 \pm 8.4\%$ (*post hoc* comparison, $P = 0.0188$) post-stress compared to males ($n = 10$–12 mice per group). Right panel mean percentage basal Aβ concentration during the 3 h of stress and post-stress period of 4–14 h. (**B**) In a separate group of mice, ISF $A\beta_{42}$ was measured by microdialysis. RS increased ISF $A\beta_{42}$ in females by 132.35% during stress, and levels remain elevated at 162.39% in the post-stress period (two-way RM ANOVA main effect of Time, $P = 0.0236$). While not statistically different from levels in males, females show a trend towards an increase from baseline compared to the post-stress period (*post hoc* comparison, $P = 0.0695$). ($n = 6$–7 mice per group) Male mice do not show differences in $A\beta_{42}$ compared to their pre-stress baseline (*post hoc* comparison, $P = 0.493$). (**C**) Female mice exposed to predator olfactory stress during microdialysis show increases in $A\beta_{40}$ compared to male mice (*t*-test of post-stress mean, $t = 3.722$, $P = 0.0204$) ($n = 3$ mice/group). (*$P > 0.05$; **$P > 0.01$; ***$P > 0.001$).

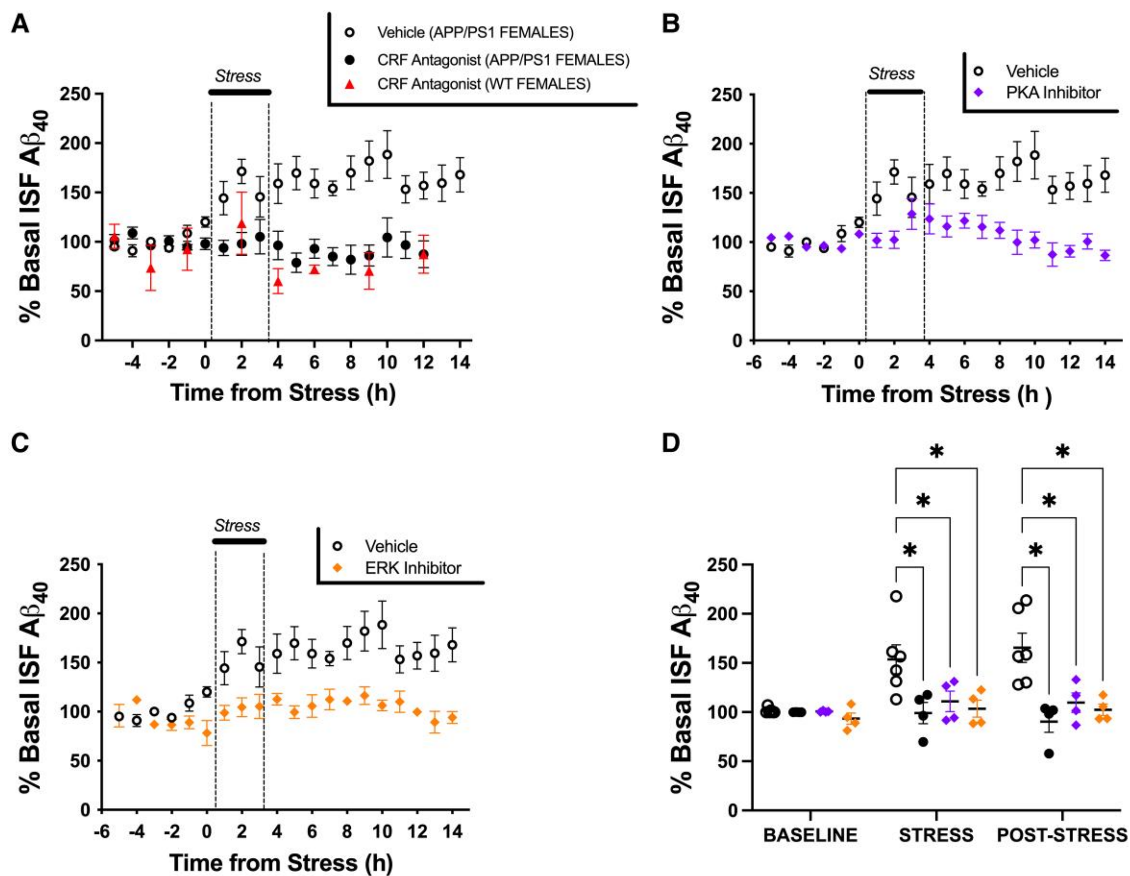

Figure 2 CRF receptor signalling drives the increase in Aβ in female mice. (**A**) APP/PS1 female mice were pretreated with a CRF-R antagonist (antalarmin, 4 nM) by reverse microdialysis directly to the hippocampus for 6 h before stress, then continually infused throughout the duration of the experiment. Inhibiting CRF-R completely blocked the effect on Aβ levels. (**B**) Similar studies inhibiting either PKA (KT5720, 6 µM) or (**C**) ERK (FR180204, 100 µM) also blocked the effect of stress on ISF Aβ in females. (**D**) Statistical comparisons across time blocks for each treatment group. Two-way RM ANOVA interaction between Treatment × Time [$F(6,28) = 5.480$, $P = 0.0007$]. *Post hoc* comparisons: Baseline: no difference. Stress: Vehicle versus CRF Antagonist $P = 0.017$, versus PKA Inhibitor $P = 0.0449$, versus ERK Inhibitor $P = 0.0197$; Post-Stress: Vehicle versus CRF Antagonist $P = 0.0037$, versus PKA Inhibitor $P = 0.0148$, versus ERK Inhibitor $P = 0.0197$. ($n = 4$–6 per group). ($^*P > 0.05$; $^{**}P > 0.01$; $^{***}P > 0.001$).

contrast, males had no significant change in ISF $A\beta_{40}$ levels during or after stress. Interestingly, 16% of males did appear to have a stress response in Aβ, but it did not significantly increase until hours after the stress was terminated (hence larger error bars from hours 7–13). We assessed changes in ISF $A\beta_{42}$ levels in response to RS and found a similar trend (Fig. 1B). To determine whether this sex difference was also present using a different form of stress, we measured ISF $A\beta_{40}$ levels in male and female mice exposed to different types of predator urine for 3 h. Again, we found ISF $A\beta_{40}$ increased in the females significantly more than in the male mice (Fig. 1C). A separate cohort of mice, males and females were exposed to RS for 3 h then immediately killed to collect tissue and measure plasma corticosterone levels, as a marker of their stress response. Importantly, both males and females show an increase in corticosterone during restraint (Supplementary Fig. 1), meaning the lack of a change in Aβ in males was not due to lack of stress.

CRF receptor signalling drives the increase in Aβ in female mice

We pretreated female APP/PS1 mice with vehicle or a CRF-R antagonist (antalarmin) to block CRF signalling by reverse microdialysis into the hippocampus for 6 h, then stressed the mice for 3 h. The antagonist completely blocked the effect RS on Aβ (Fig. 2A and D). In females, CRF-R can activate PKA and ERK therefore, we inhibited either PKA (Fig. 2B) or ERK (Fig. 2C) prior to stress using reverse microdialysis. Both inhibitors completely blocked the effect of RS on Aβ in females (Fig. 2D). PKA inhibitors had no effect on Aβ in male mice (data not shown). Importantly, this appears directly related to local CRF, as opposed to systemic corticosterone, as corticosterone administration alone does not change Aβ.[21]

Male mice lacking β-arrestin show an increase in Aβ following stress similar to female mice

We performed microdialysis with stress in wild-type (WT) C57Bl/6J mice or β-arrestin1 KO mice. Because murine Aβ levels in WT mice are lower than APP/PS1 mice, we increased the sample collection time to 180 min to detect changes in ISF Aβ. Similar to APP/PS1 mice, WT male mice also had no increase in Aβ (Fig. 3A and B). However, female WT mice and male β-arrestin1 KO mice had a significant increase in murine Aβ by ~40% compared to non-stressed mice (Fig. 3A and B). The male β-arrestin1 KO response also persisted for several hours, which was statistically the same as the female response. Replicating the stress effect in both APP/PS1 and WT mice, further emphasizes the robustness of this finding and indicates it is not an artefact of the transgene in APP/PS1 mice. To determine whether the increase in Aβ in male β-arrestin1 KO mice is dependent on CRF-R, we pretreated male BKO mice with antalarmin before stress

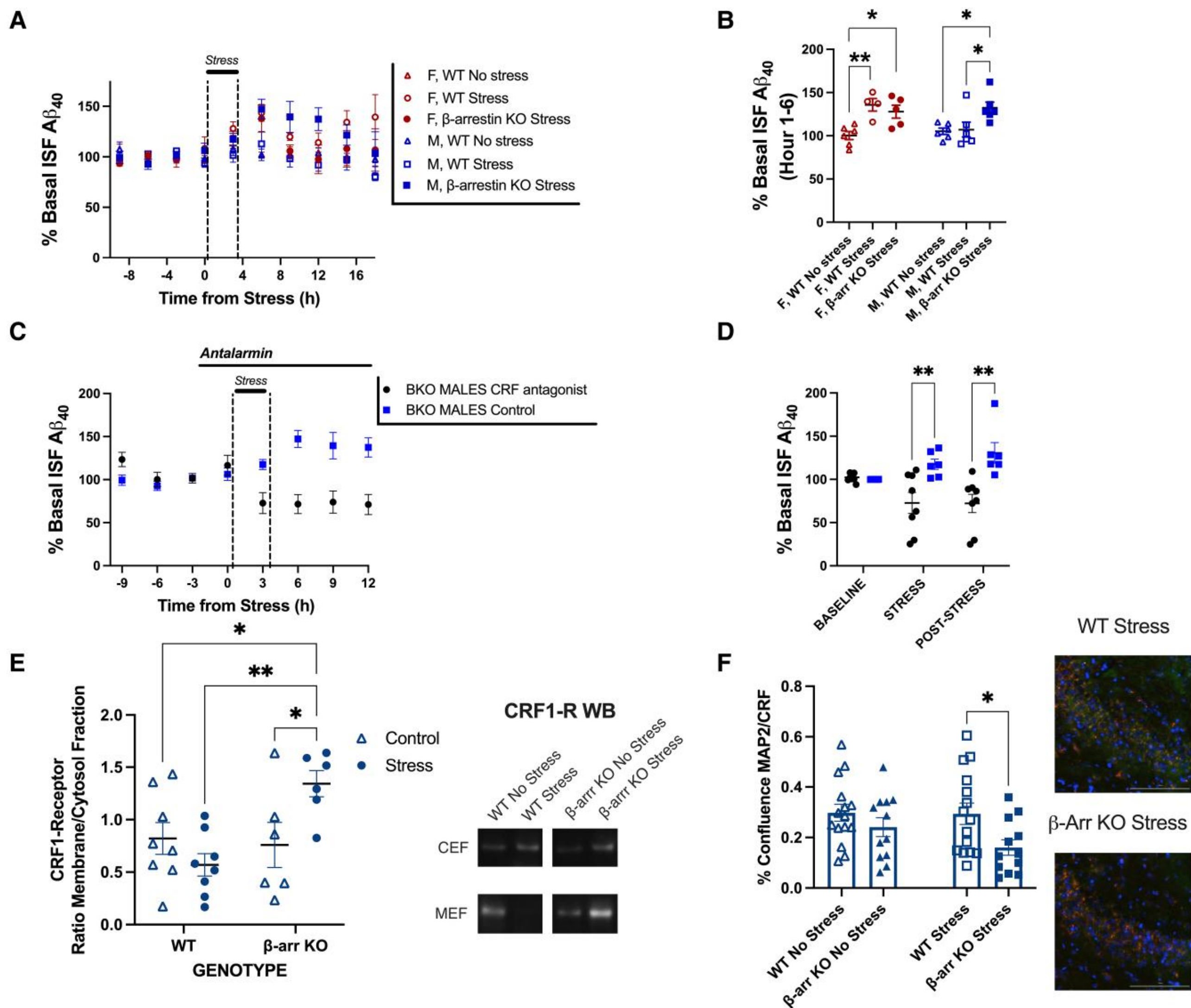

Figure 3 WT female, β-arrestin1 KO female/male mice have an increase in ISF Aβ following stress, male mice without β-arrestin1 have more CRFR1 in the membrane. (**A**) C57Bl/6J mice or β-arrestin KO mice were subjected to *in vivo* microdialysis and stress for 3 h. Female mice of both genotypes had a significant increase in ISF Aβ [one-way ANOVA $F(5,27) = 5.548$, $P = 0.0012$] by 135.8 + 7.2% (*post hoc* comparison, $P = 0.0078$, $n = 4$) and 127.9 ± 7.5% (*post hoc* comparison, $P = 0.0362$, $n = 5$), respectively, compared to unstressed females. Male WT mice had no increase in Aβ following stress ($n = 6$), however, male β-arrestin1 KO mice had an increase of 132.4 ± 6.6% (*post hoc* comparisons, $P = 0.0291$ and $P = 0.0.0497$, $n = 6$) and compared to unstressed and stressed male WT mice. (**B**) Mean percentage basal Aβ concentration from hours 1–6 from the start of stress. (**C** and **D**) Male β-arrestin KO mice were pretreated with the CRF-R antagonist, antalarmin before RS. Antalarmin blocked the effect of stress on ISF Aβ in stressed β-arrestin KO compared to untreated mice [two-way RM ANOVA interaction between Treatment × Time $F(2,24) = 6.881$, $P = 0.0043$; *post hoc* comparisons, $P = 0.0078$ during RS and $P = 0.0036$ following stress] ($n = 6$–7 mice per group). (**E**) Western blot of CRF1-R from hippocampal fractionation of WT and b-arrestin1 KO males that are stress and unstressed. The ratio of CRF1-R in the membrane increases in response to stress only in the β-arrestin1 KO males [two-way ANOVA interaction between Genotype × Stress $F(1,24) = 7.574$, $P = 0.011$]. *Post hoc* comparisons show $P = 0.023$, $P = 0.0014$ and $P = 0.0179$ compared to WT control, WT stress and β-arrestin1 KO stress, respectively, $n = 6$–8 per group). (**F**) Immunohistochemical staining shows significantly more CRF-R in the cytosol in WT male mice following RS compared to male β-arrestin knockout mice [two-way ANOVA main effect of Genotype $F(1,50) = 6.601$, $P = 0.0132$]. *Post hoc* comparisons between β-arrestin KO and WT stress groups $P = 0.0282$. (*$P > 0.05$; **$P > 0.01$; ***$P > 0.001$).

and measured ISF Aβ. Blocking CRF-R before stress completely blocked the effect of stress in male β-arrestin1 KO mice (Fig. 3C and D), similar to WT females.

In response to stress, male mice lacking β-arrestin have more CRF-R in the membrane compared to WT mice

The ratio of CRF1-R in the membrane significantly increased in response to stress in the β-arrestin1 KO males but not the WT males (Fig. 3E and F). Plasma taken at the time of sacrifice verified that the corticosterone response to stress was present, and levels were similar to those seen in other cohorts (Supplementary Fig. 2). These results indicate that β-arrestin1 is involved in the difference in signalling through the CRF1-R in males leading to the blunted increase in Aβ seen in male mice.

Discussion

These studies demonstrate that Aβ levels are altered differently between female and male mice in response to acute stress. In both APP transgenic and WT female mice there is a robust and prolonged

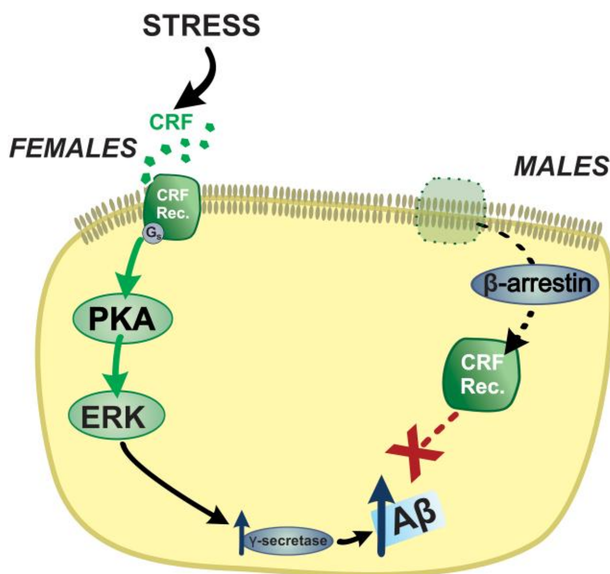

Figure 4 Stress-induced signalling pathways. *Left*: In females, during stress CRF acts within the hippocampus to activate CRF-R, PKA and ERK, probably through its G_s-coupled pathways, to increase Aβ levels. *Right*: In males, however, during stress β-arrestin internalizes the CRF-R to reduce signalling, thereby preventing the stress-induced change in Aβ.

increase in ISF Aβ during stress that persists long after the stressful event, while most males do not show a change in ISF Aβ at all. Local administration of CRF antagonist, PKA and ERK inhibitors eliminate the Aβ stress response in female mice (Fig. 2A–D) confirming dependence on local signalling of CRF within the hippocampus. A similar stress related plasma corticosterone response between male and female mice (Supplementary Fig. 1) indicates a similar CRF release between sexes in response to acute stress. Therefore, the different response of male mice relative to female mice could occur at the level of the CRF receptor. Interestingly, the CRF receptor signals differently between the sexes during stress; females activate a PKA/ERK pathway, whereas in males β-arrestin removes the receptor from the cell surface to limit signalling.[18] In β-arrestin knockout males, CRF-R is left in the plasma membrane and Aβ increases in response to stress very similar to females (Fig. 4). The basis for this sexual dimorphism in the CRF-R signalling pathway between the sexes is unknown.

CRF-R-β-arrestin has been implicated in the internalization of CRF-R from the plasma membrane[23] and has been shown to increase in response to stress in LC neurons of male, but not female rats.[18] To determine whether an association with β-arrestin functioned similarly in the hippocampus we initially attempted to deliver a β-arrestin inhibitor via reverse microdialysis, however, it was not even remotely soluble in aqueous buffers. Therefore, we subjected male β-arrestin knockout mice and WT mice to three hours of RS. The finding of an increased plasma membrane to cytosolic CRF-R ratio in male β-arrestin knockout mice, but not in WT mice in response to stress supports the notion that the primary mechanism for preventing a change in Aβ in male mice is an increase of β-arrestin associated internalization of CRF-R from the plasma membrane, limiting signalling. Further supporting this hypothesis, immunohistochemical staining shows significantly more CRF-R in the cytosol in WT male mice following stress compared to male β-arrestin knockout mice (Fig. 3F).

This study has uncovered several components that mediate the sex-dependent effect of stress on Aβ, however parts of the signalling pathways remain unknown, especially in male mice. CRF-Rs are G-protein-couple receptors that signal through G_s-proteins (typically then Src/PKA/ERK downstream) or G_q-proteins (typically PLC/PKC downstream). Downstream of all of these signalling pathways, it is possible that ISF Aβ levels could be regulated, at least in part, by synaptic activity. Synaptic activity gives rise to over 70% of Aβ found within the brain ISF.[24] CRF-Rs mediate an increased firing rate of numerous neuronal populations and this increase rate is 10–30-fold more potent in females than males.[25] We hypothesize that during stress, CRF-R signalling increases synaptic activity more so in females, which drives the elevation in ISF Aβ levels.

Reducing β-arrestin-1 and -2 decreases Aβ generation.[26,27] Although CRF1-Rs are also coupled to β-arrestin, it remains unclear whether it this is an overlapping or divergent pathway that regulates Aβ. Studies in humans show no change or a global increase in brain β-arrestin expression in AD[26,27]; however, an important variable, sex, was not controlled for. Similarly, many studies assessing β-arrestin in Alzheimer's disease mouse models were agnostic on sex.[28] While it seems that β-arrestin affects Aβ, how it specifically links to CRF-R and sex differences in that response are unclear and are a focus of this study. Sex differences in plaque load and onset of plaque deposition have been shown in some mouse models of Alzheimer's disease,[29] and it is possible that differences in the stress response contribute to these observations. Other studies have assessed the effect of stress on Alzheimer's disease, but ours are the first to determine at the cell signalling level why stress differentially affects disease-related proteins in males and females.

While there are several published studies that elegantly demonstrate the tight link between stress and Alzheimer's disease, the cellular biology underlying that link is unclear. Women being at higher risk of developing the disease is very probably multifactorial; this study strongly suggests that stress is one environmental factor that influences that greater risk. This study provides insight into how stress relates to Alzheimer's disease risk and suggests the importance of uncovering novel therapeutic avenues that may differ between males and females.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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