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Central CRF System Perturbation in an Alzheimer's Disease Knock-in Mouse Model

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Abstract

Alzheimer's Disease (AD) is often accompanied by changes in mood as well as increases in circulating cortisol levels, suggesting that regulation of the stress responsive Hypothalamic-Pituitary-Adrenal (HPA) axis is disturbed. Here, we show that APP is endogenously expressed in important limbic, hypothalamic, and midbrain nuclei that regulate HPA axis activity. Furthermore, in a knock-in mouse model of AD that expresses familial AD (FAD) mutations of both *APP* with humanized A β , and *PS1*, in their endogenous patterns (APP/hA β /PS1 animals), Corticotropin Releasing Factor (CRF) levels are increased in key stress-related nuclei, resting corticosteroid levels are elevated and animals display increased anxiety-related behavior. Endocrine and behavioral phenotypes can be normalized by loss of one copy of *Corticotropin Releasing Factor Receptor type-1* (*Crfr1*), consistent with a perturbation of central CRF signaling in APP/hA β /PS1 animals. However, reductions in anxiety and corticosteroid levels conferred by hemizyosity of *Crfr1* do not improve a deficit in working memory observed in APP/hA β /PS1 mice, suggesting that perturbations of the CRF system are not the primary cause of decreased cognitive performance.

Keywords

Corticotropin Releasing Factor; CRF; CRH; CRFR1; CRHR1; HPA axis; Alzheimer's Disease; corticosteroids; APP; Presenilin; anxiety; depression

1. Introduction

Alzheimer's Disease (AD) is characterized by the progressive loss of cognitive ability and eventual dementia. On autopsy, AD patients present with amyloid plaques and neurofibrillary tangles in addition to neurodegeneration, together considered the

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pathological hallmarks of AD. The most well characterized outcome of these neuropathologies is the progressive loss of memory, however, other brain systems have been shown to mis-function with the onset of AD. For example, AD patients exhibit elevated cortisol levels [10,23], indicating a progressive imbalance in the function of the Hypothalamic-Pituitary Adrenal (HPA) axis, the endocrine axis that is initiated by Corticotropin Releasing Factor (CRF) release by the hypothalamus and coordinates the release of corticosteroids by the adrenal glands [63]. Imbalance in HPA axis function can directly impact emotional status, responses to stress, and cognitive ability, and increased HPA axis activity in many cases accompanies depression [44]. Interestingly, changes in mood, anxiety, and depression often precede or present concomitantly with the earliest signs of memory loss or mild cognitive impairment (MCI) in AD, and are found to precede dementia in many families carrying FAD mutations in APP and PS1, leading many to propose that depression is perhaps one of the earliest signs of progressing AD pathology [14,17,30,42,45,51]. Indeed, patients with higher levels of corticosteroids suffer more rapid progression of dementia [9,10,39,67]. However, it remains unclear whether stress and increased cortisol accelerate dementia or are in fact a symptom of progressing AD pathogenesis.

While the cause of increased anxiety and corticosteroid levels in AD is still unknown, the impact of stress and corticosteroids on the development and progression of AD pathogenesis is more thoroughly understood. In transgenic animal models of AD, increased stress leads to higher A β and A β oligomer levels, increased phosphorylated tau, and accelerated amyloid plaque deposition [12,49,50,62]. This may be due to stress induced elevations in corticosteroid levels, which have been shown to have many of the same effects [6,8,20]. These studies suggest that animals pre-disposed to succumb to AD are sensitized to the effects of stress and stress hormones, which can severely provoke the progression of AD pathogenesis. However, reports on the status of stress responsive circuitry and the HPA axis at a baseline state, before stress has been given have been inconsistent. In various AD transgenic mouse models, anxiety-related behavior has been reported to be reduced [19,22,28,33], increased [13,35,58,62], or not affected [1]. Changes in corticosteroid levels have also varied in reports using different AD model animals [13,19,62]. These differences might be partially explained by differences between the heterologous promoters used to mis-express APP, which do not necessarily recapitulate the endogenous expression pattern of *APP*, and might lead to APP gain-of-function phenotypes not typically present in human AD.

To examine stress responsive neural and endocrine pathways in the context of endogenous expression of *APP* mutations, we have taken advantage of a knock-in AD mouse model (APP/hA β /PS1) in which a mutant *APP* allele carrying the disease causing Familial Alzheimer's Disease (FAD) Swedish (K670N/M671L) and London (V717F) mutations with a humanized A β sequence has replaced mouse *APP* [31]. Wildtype *PS1* has been replaced by mouse *PS1* carrying the M146V FAD mutation [21]. APP/hA β /PS1 mice display endogenous temporal and spatial expression of mutant forms of *APP* and *PS1*, and model AD in the absence of high levels of mis-expression.

2. Methods

2.1 Mouse maintenance and breeding

Mice were housed up to five per cage with *ad libitum* access to food and water in a room with a 12 h light/dark cycle in a specific pathogen-free mouse facility. All experimental procedures were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC) and performed in accordance with National Institutes of Health (NIH) guidelines. All mice used for behavioral and endocrine analysis have been

backcrossed for at least 6 generations onto a C57/B6 background. *APP^{sl/AB/sl/AB}* mice and *PS1^{M146V/M146V}* mice were bred together to generate double heterozygous *APP^{sl/AB/+}; PS1^{M146V/+}* animals which were intercrossed to generate homozygous littermates including wildtype mice with the genotype *APP^{+/+}; PS1^{+/+}* (wt), and double knock-in mice with the genotype *APP^{sl/AB/sl/AB}; PS1^{M146V/M146V}* (APP/hAβ/PS1) animals. To generate large cohorts of animals for behavioral studies, we subsequently set up wt x wt and APP/hAβ/PS1 x APP/hAβ/PS1 crosses to obtain age-matched male mice. For *Crfr1* interaction studies, APP/hAβ/PS1 mice were crossed with *Crfr1*^{+/-} mice to generate APP/hAβ/PS1; *Crfr1*^{+/-} animals. APP/hAβ/PS1; *Crfr1*^{+/-} animals were subsequently intercrossed to give APP/hAβ/PS1; *Crfr1*^{+/+}, APP/hAβ/PS1; *Crfr1*^{+/-}, and APP/hAβ/PS1; *Crfr1*^{-/-}, male offspring. Control *Crfr1*^{+/+}, *Crfr1*^{+/-}, and *Crfr1*^{-/-} animals were obtained by intercrossing *Crfr1*^{+/-} animals. The *Crfr1* allele was obtained courtesy of Wylie Vale (Salk Institute, San Diego), where it was backcrossed for more than 10 generations to C57/B6 mice.

2.2 Behavior

Elevated Plus Maze (EPM)—Mice were transferred to the testing room, which was at a light level of 700 lux and a sound level of 60db generated by a white noise generator. The EPM apparatus consists of four arms placed at right angles to each other and is elevated 50 cm from the ground. Two of the arms had 20 cm high walls (enclosed arms), whereas two had no walls (open arms). After 30 minutes of acclimatization to the testing room in their home cages, mice were placed in the center of the elevated plus maze facing the open arms and allowed to explore the maze for 10 minutes. All parameters of movement were scored by an automated ANY-maze software system (Stoelting Co., Wood Dale, IL) including time in open, center, and closed areas of the maze, as well as number and duration of each entrance into an arm. After testing, animals were returned to their home cage. Animals tested on the EPM were naïve to all behavioral testing at the time of the test. Animals were grouped depending on genotype and analyzed for statistical differences in all parameters of movement using a two-tailed Student's T-test. 2–3 month old males were used for all behavioral tests, with n=18–24 per genotype. EPM was always the first behavioral test performed and was never repeated on an individual animal.

Light-Dark (LD) box—The LD tests were done in the same room conditions described above for the EPM test. The LD apparatus has two compartments, an open transparent light chamber and a covered dark chamber. To begin the test, the animal was placed in the center of the light chamber. The animals were allowed to explore both chambers for 10min and all movements were recorded by a computer. The time spent in each chamber and the number of transitions between chambers were scored by the ANY-maze software system (Stoelting Co., Wood Dale, IL). Animals were grouped by genotype and analyzed for statistical differences in all parameters of movement.

Conditioned Fear (CF)—On the first day (training day) the mice were first transferred to a holding room next to the CF testing room in their home cages and allowed to acclimate for 30min. Mice were then transferred in a sound-attenuated cage to the testing room and placed in a testing chamber (Med Associates, St. Albans, VT). After a delay period, an auditory cue was played which was immediately followed by a mild footshock (0.7mA) for 2 sec. On the next day (testing day), mice were placed back in the same chamber for 5 min without any sound stimulus (context conditioning), during which time the movements of the mice were recorded by a video camera and the freezing frequency was scored by FreezeFrame software (San Diego Instruments, San Diego, CA). Two hours after the context test, the mice were placed in a testing box in which the environment in the chamber was changed (different floor, shape and odor). Movements of the mice were first recorded for 3 min without any sound stimulus to establish a baseline freezing level and then recorded during a 3 minute

presentation of the sound (cued conditioning). Computer generated percentages of freezing behavior were grouped according to genotype and statistically compared for significance.

Hotplate nociception test—Mice were acclimated to the testing room for 30 minutes and the hotplate was pre-warmed to 55° C before the test. Mice were placed on the hotplate one at a time. The time before the mouse showed its first hindlimb response, including jumping, shaking and licking, was recorded and statistically compared between groups.

Spontaneous Alternation T-maze—Mice were acclimated to the testing room for 30 min before the start of the test. The T-maze apparatus is a T-shaped maze with a removable door at the base of the long arm of the maze. At the beginning of each trial, the mouse was placed behind the removable door at the base of the maze. After 30 seconds, the door was removed and the mouse was allowed to explore the maze and enter one of two arms freely. Once the animal entered an arm (first choice), the arm entrance was blocked by a transparent plastic panel and the animal was restricted to the chosen arm for 5 sec. The animal was then transferred back to the base with the door closed. After a 45 second waiting period, the door was removed, and the mouse was again allowed to explore the maze and choose an arm (second choice). If on the second choice the animal enters the alternate arm from the first choice, this is scored as an alternating trial. The mice were tested on the T-maze by an observer blind to the genotype for 3 trials a day for 3 test days with a 3-day interval between each testing day. Animals were grouped by genotype and compared for statistical differences.

2.3 Corticosterone measurement

For measuring plasma corticosteroids at rest, animals were moved to a quiet procedure room and allowed to calm down and acclimatize to the room for 2 hours, during which time no entries or exits were made from the room, and all noise was minimized. After 2 hours, an experimenter entered the room and drew blood via retro-orbital eye bleed. All mice in the room were bled in under 5 minutes. For high stress corticosteroid measurements, animals were placed into restraining tubes for 20 minutes before being bled. For dexamethasone suppression studies, animals were given an injection of 30µg/kg (i.p.) dexamethasone 2 hours before being bled. Blood samples were mixed with EDTA (final concentration 2mM) and centrifuged to remove red blood cells. Plasma samples were collected and frozen before shipment to the UVA Center for Research in Reproduction Ligand Assay and Analysis Core, where corticosteroid levels were measured using a rat corticosterone RIA. For corticosteroid measurements, n=14–16 per genotype. Animals were not used in behavioral testing after being bled for corticosterone measurement.

2.4 Immunohistochemistry

IHC was performed according to standard procedures as described previously (Justice et al, 2008). Briefly, separate cohorts of 2–3 month old animals were perfused with 4% Paraformaldehyde, brains were removed and sucrose protected overnight, and then sectioned on a frozen sliding microtome at 30µM. Sections were rinsed in PBS and incubated in primary antibodies overnight in PBS with 0.4% Triton X-100, and 2% normal donkey serum, rinsed in PBS, incubated in secondary antibodies for 2 hours in PBS + 0.4% Triton X-100, rinsed in PBS and mounted on gelatin coated slides. For human tissue, we used PBS + 0.4% Triton X-100 + 5% normal donkey serum (tissue courtesy of Yong Shen). For APP immunohistochemistry, we used a rabbit monoclonal antibody directed against the C-terminal NPXY motif (APP Y188, 1:1000, Epitomics, CA), followed by incubation in biotin labeled donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA), and the vectastain elite DAB kit with nickel intensification (Vector Labs, Burlingame, CA). Brightfield images were generated using and EVOS brightfield microscope (Advanced

Microscopy Group, Bothell, WA). To detect CRF, we used rabbit anti-rat/human CRF antibodies provided by W. Vale [rC70, 1:2000; 60]. Secondary Goat anti-rabbit antibodies conjugated to Alexa-Fluor 555 (Invitrogen, San Diego) were used for detection. Images of each area were acquired on a Leica AOBs Confocal Microscope using identical setting for acquisition of all images. Quantification of the intensity of CRF immunolabeling was performed using the Nikon NIS-Elements software package by an observer blind to the genotype. A single section of a confocal stack containing the peak level of staining was used for quantification. Each image was thresholded using identical settings to remove any saturated background signal. The structure was then outlined using the Bezier tool, thereby defining an ROI. Within this outline, three non-overlapping 205 pixel square ROIs were defined. Average luminosity was calculated for each of the ROIs. The luminosity from each of the ROIs was averaged to determine a value for the intensity of CRF staining for that area. CRF staining intensities were generated for the BSTov, rostral pericommisural BST, subcommisural BST, PVN, and CeA. Because staining in the subcommisural BST was consistently low and evenly distributed, we used this area to normalize within each animal. This allowed for variance between animals in luminosity due to the quality of staining to be reduced. After normalization, luminosity scores for each area were grouped by genotype and compared for statistically significant differences using a two-tailed Student's T-test. $n=8-10$ per genotype.

2.5 Data Analysis and Statistics

For all quantifications, measurements were made blind to the genotype. Upon data analysis, the genotype of each animal was revealed. Animal scores were grouped by genotype, averaged and compared pair-wise for statistically significant differences by performing two-tailed Student's T-tests. A difference was determined to be statistically significant if $p<0.05$.

3. Results

3.1 APP is abundantly expressed in the Central Autonomic System

A highly interconnected system of brain nuclei that are coordinately activated by stress and by CRF have been collectively termed the Central Autonomic System [CAS; 52,60]. This system includes limbic structures such as the Central Nucleus of the Amygdala (CeA) and the lateral dorsal Bed nucleus of the Stria Terminalis (also known as the BSTov). The CAS additionally includes the paraventricular nucleus of the hypothalamus (PVN), which contains CRF neurons that project to the median eminence and activate the HPA axis, the Locus Coeruleus (LC) which coordinates autonomic activity, and two visceral sensory nuclei, the Parabrachial nucleus (PB) and the nucleus of the Solitary Tract (NTS). Using an antibody that specifically recognizes APP, we stained wildtype mouse brain and examined APP expression in the CAS. We found that APP is abundantly expressed in nuclei of the CAS. Specifically, we found high levels of expression in multiple regions of the BST, including the BSTov (Fig. 1A), and in the amygdala (Fig. 1B). In the amygdala, expression is highest in the Basolateral nucleus (BLA), with moderate levels of expression in the Lateral Amygdala (LA), CeA and Medial Amygdala (MeA). The PVN expresses particularly high levels of APP (Fig. 1C), while the PB, LC and NTS all express moderate to high levels of APP (Fig. 1D, E). Labeling was specific for APP because in APP null mutant animals [70], all staining in the PVN (Fig. 1F) and other areas (data not shown) is absent. Using the same antibody on human amygdala sections from non-diseased brains, we saw prominent APP labeling of neurons in the Basolateral and Cortical nuclei with lower levels of staining in the Central nucleus (Fig. 1G-I), similar to the APP expression pattern observed in the mouse amygdala. Many transgenic AD model animals express mutant forms of APP from heterologous promoters which often lack expression in these important stress responsive nuclei. Endogenous expression of APP in neurons of key limbic, autonomic, and

sensory nuclei suggest that APP may be important to the function of these neurons, and that disease causing mutations may directly impact the excitability of the CAS.

3.2 Increased central CRF levels in APP/hA β /PS1 animals

Release of corticosteroids is initiated by the release of CRF into the portal system by hypophysiotropic CRF neurons which reside in the PVN [63]. CRF also functions centrally, where it modulates activity of stress responsive neuronal circuitry and drives anxiety-related behavior [32,60]. We examined the expression of CRF in key stress responsive nuclei by performing immunohistochemistry for CRF peptide and quantifying relative fluorescence levels. Because staining intensity may vary depending on the quality of fixation of the tissue and the efficacy of staining, within each sample we normalized fluorescence levels with CRF levels in the medial-ventral BST (BSTmv) which expresses moderate to low levels of CRF, and where CRF expression is relatively consistent between animal groups (data not shown). CRF is expressed at the highest levels in the BSTov, PVN, and CeA (Fig. 2). Within the BSTov, which has been proposed to be critical for the expression of chronic anxiety [65], CRF levels were found to be significantly higher in APP/hA β /PS1 animals compared to wildtype controls (Fig 2A,B; $p < 0.05$). Similarly, in the PVN we saw that CRF levels were higher in APP/hA β /PS1 animals compared to wildtype controls (Fig. 2A,B; $p < 0.05$). In the CeA, we found no statistical difference in CRF levels between APP/hA β /PS1 and wildtype groups (Fig. 2A,B, $p = 0.32$). We also measured CRF levels in APP single knock-in and PS1 single knock-in animals, and found that CRF is elevated in the BSTov of APP single knock-ins but is at wildtype levels in PS1 single knock-in animals, suggesting that this increase is primarily due to mutations in *APP* (Figure S1E). Higher levels of CRF in key limbic, stress responsive, nuclei such as the BSTov and PVN suggest that these circuits are perturbed by *APP*, which led us to investigate endocrine and behavioral outputs of this circuitry.

3.3 Elevated resting corticosterone levels in APP/hA β /PS1 animals

Given elevated CRF levels in important stress responsive central nuclei including the PVN, we next measured corticosteroid levels, which have been shown to be elevated in elderly patients with MCI in the early stages of AD progression [10,23]. We found that APP/hA β /PS1 animals have higher resting levels of circulating corticosteroids compared to control animals (Fig. 2C), indicating basal activity of the HPA axis is perturbed. Next, we tested corticosteroid levels after 20 minutes of restraint stress when corticosteroid levels peak in response to this stressor. We found that APP/hA β /PS1 animals on average have elevated peak corticosteroid levels compared to wildtype animals, however, this failed to reach statistical significance (Fig. 2D; $p = 0.09$). Finally, we administered dexamethasone, a potent glucocorticoid receptor agonist which suppresses corticosterone release by inhibiting CRF and Adenocorticotrophic Hormone (ACTH) release, and measured circulating corticosterone levels two hours later. In wildtype and APP/hA β /PS1 animals we saw an equivalent reduction in circulating corticosterone levels, indicating that the efficacy of corticosteroid negative feedback on the HPA axis is normal in APP/hA β /PS1 animals (Fig 2E).

3.4 Enhanced anxiety-related behavior in APP/hA β /PS1 animals

Given changes in CRF levels in key stress responsive nuclei, and elevated resting corticosteroid levels, we next examined anxiety-related behavior in APP/hA β /PS1 animals. We saw that on the Elevated Plus Maze (EPM), which measures anxiety-related behavior by determining how much time an animal is willing to investigate exposed arms of an elevated plus maze versus how much time the animal stays in the closed arms of the maze [46], APP/hA β /PS1 animals spent significantly more time in the closed arms and significantly less time on the open arms of the maze compared to wildtype control animals, indicating higher levels of anxiety (Fig. 3A). In addition, APP/hA β /PS1 animals made fewer entries into the open

arms and navigated less distance on the open arms compared to wildtype animals, indicating less willingness to explore the exposed arms (Fig. 3B, C), a sign of increased anxiety [46]. APP/hA β /PS1 animals also displayed elevated anxiety-related behavior on the Light-Dark box (LD) test. Similar to the EPM, the LD tests how much time the animal spends in the open and lighted side or in the closed and covered area of a two chambered box [4]. APP/hA β /PS1 animals spent significantly more time in the closed portion of the box, and significantly less time in the open side (Fig. 3D). APP/hA β /PS1 animals made fewer entries to the light side of the box, and spent on average more time on each visit to the dark side (Fig. 3E, F), suggesting increased hiding behavior and reduced exploring, which suggests higher levels of anxiety. Animals carrying only *APP* mutations or *PS1* mutations were also tested for anxiety-related behavior. APP single knock-in animals displayed decreased open time and increased closed time on the EPM, similar to APP/hA β /PS1 animals, while PS1 single knock-in animals did not display the anxiety phenotype (Fig. S1A,B). Additionally, we tested APP/hA β /PS1 animals that were over 12 months of age and found that the anxiety phenotype persists at this stage, after the commencement of amyloid plaque deposition [Fig. S1C,D; 15]. The observed increases in anxiety-related behavior displayed by APP/hA β /PS1 animals is consistent with overactive central CRF signaling and increased corticosteroid levels [24].

To further examine the anxiety phenotype of APP/hA β /PS1 mice, we tested the ability of mice to pair a painful footshock with a sound or with the context in which they received the shock. This Conditioned Fear (CF) paradigm has been used extensively to dissect the function of the amygdala in establishing the association between a sound and a painful stimulus [34]. 24 hours after two exposures to a paired sound and footshock, animals were returned to the original box in which they were shocked, or to a different box with altered cage floor, walls, and scent. Freezing was measured in the context of the shock in the absence of the sound (contextual fear), or in the new context during presentation of the sound (cued fear). APP/hA β /PS1 mice displayed increased freezing in the context in which they received the shock compared to wildtype animals but displayed similar levels of freezing to wildtype animals in response to the sound (Fig 4A). Interestingly, during the cued trial, we measured freezing levels in the 3 minutes immediately before presentation of the sound, and found that APP/hA β /PS1 animals displayed increased baseline freezing levels, suggesting that higher levels of amygdalar activity are present in the absence of the context or the sound (Fig 4B). Increased baseline freezing was not present before the animals were shocked (Fig 4B, pre-shock). Changes in baseline freezing are not likely due to a pain threshold difference because we detected no statistical difference in nociception by the hotplate assay (3.68 vs. 3.92 seconds to twitch, $p=0.38$). Increased anxiety-related behavior on these three behavioral paradigms indicates a perturbed stress-reactive circuitry, due in part to increased levels of CRF, which enhance fear and anxiety-like responses.

3.5 *Crfr1* hemizygosity reverses elevated corticosterone levels and anxiety-related behavior in APP/hA β /PS1 animals

CRFR1 is required for the activation of ACTH release by pituitary corticotropes, and also mediates anxiogenic actions of CRF in the central nervous system [48,56]. Therefore, we intercrossed the APP/hA β /PS1 double knock-in mouse with a null allele of *Crfr1* to generate APP/hA β /PS1; *Crfr1*^{+/+} animals, APP/hA β /PS1; *Crfr1*^{+/-} animals that lack one copy of *Crfr1*, and APP/hA β /PS1; *Crfr1*^{-/-} animals that are null for *Crfr1*, and measured resting and peak circulating corticosteroid levels. Loss of one copy of *Crfr1* (*Crfr1*^{+/-}) in APP/hA β /PS1 animals returned resting corticosteroid levels to levels comparable to wildtype animals (Fig. 5A, $p<0.001$), suggesting that a reduction in CRFR1 signaling can reverse the HPA axis perturbation seen in APP/hA β /PS1 animals. We did not see a decrease in the resting corticosteroid levels in *Crfr1*^{+/-} control animals, consistent with previous work indicating

that heterozygous *Crfr1* mutations do not decrease resting corticosteroid levels [Fig. 5A; 36,61]. This suggests that reduced CRF signaling only mitigates corticosteroid release in the case of CRF system perturbation, as we see in the APP/hA β /PS1 mouse. We also measured corticosteroid levels as they peak in response to restraint stress. We saw no difference in peak levels of corticosteroids in heterozygous *Crfr1* animals (*Crfr1*^{+/-}) of either genotype, suggesting that the HPA axis can be activated to a similar degree in response to stress (Fig. 5B). We also measured resting and peak corticosteroid levels in APP/hA β /PS1 animals carrying homozygous mutant copies of *Crfr1* (*Crfr1*^{-/-}). As has been shown previously [56,61], both resting and peak corticosteroid levels were at or below the limits of detection, indicating a near complete block of HPA axis activity in *Crfr1* homozygous mutants (not shown).

Next, we measured anxiety-related behavior on the EPM in cohorts of APP/hA β /PS1; *Crfr1*^{+/+} and APP/hA β /PS1; *Crfr1*^{+/-} animals, along with cohorts of *Crfr1*^{+/+} or *Crfr1*^{+/-} animals as controls. Animals homozygous mutant for *Crfr1* (*Crfr1*^{-/-}) were excluded from the behavioral analysis because of potential non-specific effects of chronically low corticosteroid levels. In APP/hA β /PS1 animals wildtype for *Crfr1* (*Crfr1*^{+/+}), we again saw an increase in anxiety-related behavior measured as an increase in time spent in the closed arms (Fig. 5C, *p*<0.01) and a decrease in time spent in the open arms (Fig. 5D, *p*<0.01). In APP/hA β /PS1 animals heterozygous for *Crfr1* (*Crfr1*^{+/-}), closed time decreased compared to APP/hA β /PS1; *Crfr1*^{+/+} animals (Fig. 5C, *p*<0.01). Similarly, APP/hA β /PS1; *Crfr1*^{+/-} animals displayed increased open time compared to APP/hA β /PS1; *Crfr1*^{+/+} animals (Fig. 5D, *p*<0.05). These results suggest that reducing CRF signaling levels by removing a single copy of *Crfr1* can reduce anxiety levels in APP/hA β /PS1 animals without changing anxiety levels in a wildtype background. However, while anxiety as measured by closed arm time was equivalent between *Crfr1*^{+/-} and APP/hA β /PS1; *Crfr1*^{+/-} (Fig. 5C), APP/hA β /PS1; *Crfr1*^{+/-} animals still displayed more anxiety than *Crfr1*^{+/-} controls measured by the time they spent on the open arms (Fig. 5D, *p*<0.05). Therefore, even in the context of *Crfr1* hemizygosity, where resting corticosteroid levels have returned to normal levels (Fig., 5A), APP/hA β /PS1 animals have elevated anxiety, suggesting additional central anxiogenic mechanisms that act in the APP/hA β /PS1 mice.

3.6 *Crfr1* hemizygosity does not improve working memory in APP/hA β /PS1 animals

To determine whether high anxiety levels in APP/hA β /PS1 animals affect cognitive performance, we tested working memory in APP/hA β /PS1 animals with and without *Crfr1* mutations using the spontaneous alternation T-maze test. Rodents spontaneously alternate between two arms of a T-maze in consecutive trials [11]. Working memory is required for the animal to successfully alternate because a memory of which arm was selected on the previous trial must be maintained for a duration period between trials. We tested APP/hA β /PS1 animals on the T-maze and found that they display decreased alternation compared to wildtype control animals, suggesting reduced working memory (Fig. 6). In APP/hA β /PS1 animals carrying a heterozygous mutation of *Crfr1* (*Crfr1*^{+/-}), alternation rates were similar to APP/hA β /PS1; *Crfr1*^{+/+} animals (Fig. 6), suggesting that working memory is not improved by the reduction in the levels of anxiety-related behavior conferred by loss of one allele of *Crfr1*.

4. Discussion

4.1 Expression of APP in CAS nuclei

APP is highly expressed in the central nervous system as well as in many peripheral tissues [71]. Early immunohistochemical studies in rat brain revealed that although APP is expressed in all neuronal populations, the expression level of APP varies in different brain

regions [7,43]. We took advantage of a highly specific APP antibody and found high levels of APP expression in important stress-related areas, particularly the BST, Basolateral amygdala (BLA), and PVN. Expression of APP in these nuclei suggests that APP may function directly in limbic pathways to modulate the activity or connectivity of neurons that respond to stress. Previously used models of AD have relied on transgenic expression of APP from heterologous promoters. One of the most commonly used APP transgenic AD mouse model animals expresses mutant forms of APP from a hamster PrP promoter [tg2576; 27]. Although this promoter is believed to be expressed in all neurons, transgenic studies using the PrP promoter to express marker proteins have found that expression is exaggerated in the cortex and hippocampus and there is little or no expression in the amygdala, hypothalamus, and other nuclei of the CAS [5]. Other transgenic model animals have used the Thy-1 or PDGF promoters, and have been selected for high levels of cortical expression, but may lack significant expression in limbic structures [18,41,59]. Consistent with different patterns of expression of mutant APP in transgenic models, reported anxiety-related phenotypes in these models have been varied [1,13,19,22,28,33,35,58,62]. In the APP/hA β /PS1 knock-in AD model animal, endogenous expression of mutant forms of APP in key nuclei of the CAS may lead to a direct perturbation of stress responsive circuits, perhaps more accurately modeling how the stress system becomes disrupted in human cases of AD.

4.2 Direct and indirect actions of APP/PS1 mutations on HPA axis activation

APP/hA β /PS1 animals have elevated central CRF levels and elevated circulating corticosteroid levels at a young age, before any amyloid plaque deposition has taken place. How do *APP* and *PS1* mutations lead to increased CRF release and HPA axis activity? One possibility is that altered APP and PS1 function leads to changes in the excitability of neural circuits that regulate HPA axis activity. Loss of APP function has been shown to increase levels of the L-type calcium channel subunit CaV1.2, leading to increased excitability of GABAergic hippocampal neurons [69]. FAD mutations in *APP* might disrupt APP function to alter the excitability of the predominantly GABAergic CeA or BST. The excitability of neurons might also be perturbed by elevated levels of toxic species of A β being produced in cells of the CAS. FAD mutations in *APP* and *PS1* skew cleavage of APP towards production of the more amyloidogenic and toxic A β 42 species [3]. Oligomers of A β 42 have been shown to block long-term potentiation and synapse remodeling and to interfere with NMDA receptor function and glutamate uptake [16,38,54,55,66]. Production of these toxic species in CAS nuclei would directly alter regulation of the HPA axis. In support of an A β based model of stress system perturbation, APP single knock-in animals displayed a similar anxiety phenotype and elevated CRF levels, whereas this phenotype was not present in PS1 single knock-in animals (Fig. S1A,B, E). PS1 knock-in mutations confer only a mild increase in total A β levels in this genetic context, further supporting an A β toxicity model [37]. Alternatively, *APP* and *PS1* FAD mutations might lead to reduced inhibition of the HPA axis by hippocampal and cortical structures, thereby increasing corticosteroid levels and promoting anxiety-related behavior. However, when mutant forms of APP are transgenically expressed selectively in the entorhinal cortex, leading to amyloid plaque formation in the hippocampus, anxiety-related behavior is reduced, suggesting that cortical and hippocampal APP mis-expression alone does not drive anxiety but in fact might reduce anxiety levels [22]. Consistent with this finding, tg2576 mice display reduced anxiety-related behavior at an advanced age [33]. Increased levels of corticosteroids have been reported in tg2576 and other transgenic AD mouse model animals, suggesting that high levels of mis-expression can perturb the HPA axis, although whether this occurs through the same mechanism as in the APP/hA β /PS1 mutant animal remains unclear [13,50,62].

4.3 The role of CRF in anxiety-related behavior and AD pathogenesis

We have shown that CRF levels are increased in APP/hA β /PS1 mice. Increased CRF can have a direct impact on AD pathogenesis. CRF and stress have been shown to cause elevated release of A β [29]. CRF also acts on hippocampal neurons to increase levels of tau phosphorylation [49]. Whether *APP* and *PS1* mutations are the initial cause of increased CRF release or serve to exacerbate stress responses remains an open question. We abrogated activity of the CRF system by genetically removing copies of *Crfr1* in the background of APP/hA β /PS1 mutations and saw that removing a single copy of *Crfr1* reduced anxiety-related behavior and normalized resting corticosteroid levels, while hemizygosity for *Crfr1* alone does not alter resting corticosteroid levels [Fig. 5A; 36,61]. Therefore, reduction of CRF signaling through CRFR1 alleviates perturbation of the CAS by early stage AD pathogenesis caused by mutations in *APP* and *PS1*, consistent with elevated CRF and corticosteroid levels in part causing altered anxiety-related behavior. However, we cannot exclude the possibility that a reduction of CRF signaling functions in parallel to reduce anxiety-related behavior, caused by APP/PS1 mutations through CRF independent mechanisms. High levels of stress and corticosteroids can also negatively impact cognitive performance. Subjecting mice to a mild chronic stress protocol has been shown to produce depressive-like features, increase resting corticosteroid levels, and decrease cognitive performance [2,57,68]. However, we do not see a benefit to cognitive performance when we reduce anxiety levels in APP/hA β /PS1 mutants by including a heterozygous *Crfr1* mutation (Fig. 6). This suggests that observed reductions in working memory in APP/hA β /PS1 mice are not caused by perturbations to the stress circuitry. We would predict that events affecting working memory are localized to the hippocampus, because this structure is required for the spontaneous alternation working memory task [11]. It remains formally possible that disruptions at the level of the hippocampus also account for activation of the HPA axis and elevated anxiety-like behavior, as the hippocampus provides important inhibitory tone to stress responsive limbic circuits. However, given high levels of expression of APP in these circuits, and reductions in anxiety-related behavior observed when APP mutants are selectively mis-expressed in hippocampal pathways [22], we favor a scenario in which mutant forms of *APP* and *PS1* directly activate these circuits.

4.4 CRF independent anxiogenic mechanisms

Although we can normalize resting corticosteroid levels in APP/hA β /PS1 animals by removing one copy of *Crfr1*, this did not normalize all aspects of anxiety-related behavior. Additionally, removing both copies of *Crfr1* (*Crfr1*^{-/-}), which blocks HPA axis function and renders the animals profoundly corticosteroid deficient [56], displayed mild increases in anxiety-related behavior, perhaps due to reduced robustness and sickness (data not shown). Therefore, there are most likely other, CRF-independent, mechanisms driving increases in anxiety. One potential source of this anxiogenic activity is LC. The LC has been shown to degenerate preferentially in AD, and LC function has been shown to be perturbed in AD model animals [25,40]. Another alternative is anxiogenic actions of CRF through its second receptor, CRFR2 [47], which is expressed in distinct subsets of neurons from CRFR1 [64]. CRFR2 has been implicated in some CRF mediated anxiogenic activity through actions in the lateral septum, which maintains strong connectivity with the CAS [26]. Identifying this additional source of anxiety may further our understanding of the mechanism by which the central CRF system becomes perturbed, due to the high degree of cross-talk between different stress responsive circuits.

4.5 The influence of the HPA axis on AD susceptibility and progression

Activation of the HPA axis causes the release of corticosteroids which have been shown to be detrimental to the health of neurons [53]. Because of this and other findings, much of the focus on the role of corticosteroids in AD has been on the negative influence that increased

levels of corticosteroids have on amyloid plaque formation. For example, injection of dexamethasone, a glucocorticoid receptor agonist, has been shown to increase APP and BACE1 expression, increase A β production, and increase tau pathologies in the 3x-FAD AD mouse model [20]. In the AD mouse model tg2576, isolation stress leads to elevated corticosteroid levels and exacerbates plaque formation [12,13]. Recently, it has been shown that chronic mild stress in the 3x-FAD model animal can lead to prolonged exacerbation of features of anxiety including anxiety related behavior and elevated corticosteroid levels, leading to higher A β levels and decreased BDNF [50]. We have described an endogenous APP/PS1 knock-in mouse model with heightened stress responses before any stress manipulations, suggesting that ongoing AD pathogenesis sensitizes these mice to perturbation of central and endocrine stress circuitry. Enhanced stress responses can then impact AD progression through mechanisms involving elevated CRF, corticosteroids, and other consequences of elevated stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

APP is abundantly expressed in nuclei of the Central Autonomic System (CAS). Immunohistochemical staining for APP reveals high levels of expression in the Bed Nucleus of the Stria Terminalis (BST; A), Amygdala (LA-lateral, BLA-Basolateral, CeA-Central, MeA-Medial, B), Paraventricular Nucleus of the Hypothalamus (PVN; C), , Locus Coeruleus (LC) and Parabrachial Nucleus (PB; D), and the nucleus of the Solitary Tract (NTS; E). (F) Lack of staining in a section of the PVN from an APP null mutant (APP^{-/-}) demonstrates the specificity of the antibody used against APP. A (G, H, I) Staining of human amygdala sections for APP reveals prominent staining in the Basolateral (G), Cortical (H) and Central (I) nuclei. Scale Bar: A–F = 200 μ m; G–I = 100 μ m

Figure 2.

APP/hAβ/PS1 animals have increased CRF levels and increased circulating corticosterone. (A) CRF labeling in wt (top row) and APP/hAβ/PS1 (second row) shows increased CRF levels in the BSTov and the PVN of APP/hAβ/PS1 animal, while CRF levels in the CeA were comparable between genotypes. Scale bar = 100 μm. (B) Quantification of fluorescence levels revealed a statistically significant difference in signal intensity between wildtype (white bars) and APP/hAβ/PS1 (black bars) in the BSTov ($p < 0.05$) and the PVN ($p < 0.05$). (C, D) Corticosterone was measured in blood samples from wildtype (white bars) and APP/hAβ/PS1 mice (black bars) at rest (C) and at the peak of corticosterone release in response to stress (D). APP/hAβ/PS1 had higher resting corticosterone levels ($p < 0.05$) and similar peak corticosterone levels. (E) After injection with dexamethasone, both wildtype and APP/hAβ/PS1 animals suppressed corticosterone release to a similar degree.

Figure 3.

APP/hAβ/PS1 animals display elevated anxiety-related behavior. Results of behavioral testing of wt animals (white bars) and APP/hAβ/PS1 animals (black bars) on the Elevated Plus Maze (EPM; A,B,C) and Light-Dark box (LD; D,E,F). (A) On the EPM, APP/hAβ/PS1 spent less time on the open arms ($p < 0.01$) and more time on the closed arms of the maze ($p < 0.05$). (B) APP/hAβ/PS1 animals made fewer entries into the open arms ($p < 0.05$). (C) APP/hAβ/PS1 animals traveled less distance on the open arms of the maze ($p < 0.001$). (D) In the LD box, APP/hAβ/PS1 animals spent less time in the light area of the box ($p < 0.01$), and more time in the dark area ($p < 0.01$). (E) APP/hAβ/PS1 animals made fewer entries into the light area ($p < 0.05$) and fewer entries into the dark area ($p < 0.05$). (F) On each visit to the dark area of the box, APP/hAβ/PS1 animals stayed for a longer period of time ($p < 0.05$).

Figure 4.

APP/hAβ/PS1 animals display anxiety-like phenotypes in conditioned fear testing. (A) When placed back in the context where they were shocked (Context), APP/hAβ/PS1 animals displayed higher levels of freezing ($p < 0.05$). In response to the sound that was paired with the shock (Cue), APP/hAβ/PS1 did not have higher rates of freezing compared to wildtype animals. (B) Both wildtype (white bars) and APP/hAβ/PS1 (black bars) animals had very low freezing rates before being shocked in the conditioned fear box (Pre-Shock). After receiving sound-shock pairings, in a novel context but before the sound was presented (Post-Shock), APP/hAβ/PS1 animals displayed higher freezing rates ($p < 0.05$).

Figure 5.

Crfr1 hemizygosity normalizes corticosteroid levels and reduces anxiety-related behavior. Circulating corticosteroid levels were measured at rest (A) and at peak levels of stress (B) in wildtype control (white bars) and APP/hAβ/PS1 animals (black bars) carrying wildtype (*Crfr1*^{+/+}) or heterozygous (*Crfr1*^{+/-}) alleles of *Crfr1*. (A) At rest, APP/hAβ/PS1 animals have higher resting corticosterone levels. This increase is normalized after removal of one copy of *Crfr1* (*Crfr1*^{+/-}). Loss of one copy of *Crfr1* has no impact on resting corticosterone levels in a wildtype background. (B) At peak stress, wildtype and APP/hAβ/PS1 animals carrying wildtype or heterozygous mutant alleles of *Crfr1* have similar peak corticosterone levels. (C,D) Anxiety-related behavior was measured as time spent in the closed arms (C) and open arms (D) of the EPM, in animals wildtype (white bars) or mutant for APP/hAβ/PS1 (black bars). (C) APP/hAβ/PS1 animals spent more time in the closed arms of the EPM compared to wildtype animals ($p < 0.01$). When one copy of *Crfr1* was mutant (*Crfr1*^{+/-}), APP/hAβ/PS1; *Crfr1*^{+/-} animals spent less time in the closed arms compared to APP/hAβ/PS1; *Crfr1*^{+/+} animals ($p < 0.01$), displaying similar closed arm times to *Crfr1*^{+/-} control animals. (D) APP/hAβ/PS1 animals wildtype for *Crfr1* (*Crfr1*^{+/+}) spent less time on the open arms ($p < 0.01$) compared to wildtype animals. When one copy of *Crfr1* is mutant (*Crfr1*^{+/-}), APP/hAβ/PS1; *Crfr1*^{+/-} animals increased the time spent on the open arms ($p < 0.05$). *Crfr1*^{+/-} control animals still spent more time on the open arms than APP/hAβ/PS1; *Crfr1*^{+/-} animals ($p < 0.05$).

Figure 6.

Reduced cognitive performance is not reversed by *Crfr1* hemizygosity. Working memory was measured using a spontaneous alternation task in wildtype (white bars) and APP/hAβ/PS1 (black bars) animals. APP/hAβ/PS1 animals displayed reduced alternation rates compared to wildtype animals ($p < 0.05$). Removal of one copy of *Crfr1* in APP/hAβ/PS1 animals did not increase alternation rates. APP/hAβ/PS1; *Crfr1*^{+/-} animals alternated at a similar frequency as APP/hAβ/PS1; *Crfr1*^{+/+} animals. Similarly, *Crfr1*^{+/-} control animals alternated at a similar frequency as *Crfr1*^{+/+} wildtype animals.