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## Altered Expression of Alzheimer's Disease-Related Proteins in Male Hypogonadal Mice

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Age-related depletion of estrogens and androgens is associated with an increase in Alzheimer's disease (AD) brain pathology and diminished cognitive function. Here we investigated AD-associated molecular and cellular changes in brains of aged hypogonadal (*hpg*) male and female mice. *hpg* Mice have a spontaneous, inactivating genetic mutation in the *GnRH* gene resulting in life-long deficiency of gonadotropins and gonadal sex hormones. Western blot analysis revealed low levels of amyloid precursor protein and high levels of presenilin 1, amyloid precursor protein C-terminal fragment, and  $\beta$ -amyloid 42 in brains of aged male, but not female, *hpg* mice. Changes were confined to the hippocampus and were not evident in the cerebellum or other brain tissues. Male *hpg* mice tended to have lower levels of IL-1 $\beta$  protein than male littermate controls. Immunohistochemical staining of the basal forebrain revealed that male *hpg* mice had lower choline acetyltransferase levels per neuron compared with controls. These AD-like changes specific to male *hpg* mice supports a link between androgen depletion and the development of AD pathology. (*Endocrinology* 153: 2789–2799, 2012)

**A**ge-related decline in circulating sex hormones has diverse effects on virtually all tissues. In the brain, low levels of circulating sex hormones have been linked to decreased cognitive function in males and females (1–4), as well as increased neurodegeneration (5–7). Low levels of endogenous sex hormones may also be a risk factor for Alzheimer's disease (AD) in both males and females (8–10). Men with AD are more likely to have lower levels of circulating and brain testosterone than cognitively normal age-matched controls (11–13). Likewise, females with AD also have lower levels of circulating and brain estradiol than cognitively normal age-matched controls (10, 14).

The two main pathological hallmarks of AD are senile plaques and neurofibrillary tangles, which are primarily composed of  $\beta$ -amyloid ( $A\beta$ ) and hyperphosphorylated  $\tau$ , respectively.  $A\beta$  is produced by amyloidogenic processing

of amyloid precursor protein (APP). APP is initially cleaved by  $\beta$ -site APP cleaving enzyme (BACE) to produce APP-C-terminal fragment (APP-CTF), which is then cleaved by  $\gamma$ -secretase to produce  $A\beta$  of various lengths,  $A\beta_{40}$  and  $A\beta_{42}$  being the most common forms (reviewed in Ref. 15). Chemical depletion of gonadal hormones in men being treated for prostate cancer results in increased plasma  $A\beta$  levels (16). Animal studies have also shown that depletion of androgens results in increased  $A\beta$  (17–19) and that testosterone is protective against hyperphosphorylated  $\tau$  *in vivo* (20). Similarly, depletion of estrogens also results in increased  $A\beta$  in some (21–23) but not all studies (24–27). Overall, these results suggest that depletion of sex hormones may result in increased AD-like brain pathology, which could provide the link behind the in-

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Abbreviations:  $A\beta$ ,  $\beta$ -Amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; APP-CTF, APP-C-terminal fragment; BACE,  $\beta$ -site APP-cleaving enzyme; ChAT, choline acetyltransferase; GDX, gonadectomy/gonadectomized; GFAP, glial fibrillary acidic protein; *hpg*, hypogonadal; P450scc, P450 side-chain cleavage; PAS, periodic-acid Schiff; PS1, presenilin 1.

creased risk for AD and reduced levels of circulating sex hormones.

Determining the specific effects of individual sex hormones in the brain is difficult using pharmacological means due to the closed-loop negative-feedback mechanisms operating the hypothalamic-pituitary-gonadal axis that produce confounding reflex effects to administration of exogenous steroids. The alternative of an open loop (*i.e.* castration) can only be achieved postnatally and may involve nonphysiological patterns of hormone delivery. Most neuroscience research has examined the brain effects of sex hormone depletion using gonadectomized (GDX) animals with or without administration of exogenous sex steroids. Interpreting these studies is influenced by systematic design differences including the age at GDX and mode of hormone delivery. An alternative to pharmacological methods to evaluate sex steroid effects is the use of genetic animal models to eliminate sex steroid effects.

This study used the hypogonadal (*hpg*) mouse model as an alternative to GDX animals to examine the effects of life-long sex steroid depletion. These mice have a spontaneous, inactivating genetic mutation involving a major deletion (28) in the *GnRH1* gene, preventing GnRH production and resulting in lifelong reproductive immaturity with low blood levels of gonadotropins (29, 30) and gonadal sex steroids (31). Although extensively studied to examine reproductive function (32), to date there has been little focus on age-related morphological or molecular changes in the brains of these animals with lifelong deficit of reproductive hormones. Thus the focus of this study was to determine the effect of lifelong reproductive hormone depletion on certain molecular pathways involved in AD including amyloidogenic processing of APP, choline acetyltransferase (ChAT) expression, and mediators of neuroinflammation. Specific brain regions such as the hippocampus are primarily affected in AD, whereas other brain regions such as the cerebellum are comparatively spared; therefore, molecular changes associated with AD were examined in both of these regions of aged animals of both genders.

## Materials and Methods

### Animals

Both *hpg* and background (C3H/J) wild-type littermate mice were obtained from the ANZAC Research Institute in Sydney, Australia and genotyped as described previously (33). The age of *hpg* and wild-type mice when euthanized ranged from 12–19 months (Table 1). Of the 34 mice used, 31 were aged between 17 and 19 months. Only three mice, which were all *hpg* male mice, were killed at 12 months of age. All animal experimentation was approved by the Animal Welfare Committee of the Sydney South West Area Health Service as well as the Animal Ethics Committee of the University of Western Australia and conformed to national guidelines for animal experimentation.

### Antibodies

The APP monoclonal antibody C1/1.6, directed against the last 20 residues of APP, was used to detect full-length APP and APP-CTF (gift from Dr. Paul Mathews, Nathan Kline Institute, New York, NY). Presenilin 1 (PS1) was detected using antibody 14, a rabbit polyclonal antiserum specific for amino acids 3–15 of PS1 (gift from Professor Sam Gandy, Nathan Kline Institute). Mouse APP/A $\beta$  was detected using the Rb194 rabbit antiserum specific for amino acids 3–16 of mouse A $\beta$  (gift from Dr. Pankaj Mehta, New York State Institute for Basic Research, New York, NY). Antibodies against glial fibrillary acidic protein (GFAP) (Sigma Chemical Co., St. Louis, MO), ChAT (Chemicon, Temecula, CA), P450 side-chain cleavage (P450sc; Chemicon) and apolipoprotein E (apoE; Millipore Corp., Bedford, MA) were all commercially purchased.

### Tissue processing

Mice were separated into two groups, one for immunohistochemistry and morphological analysis and one for protein quantification using Western blot, ELISA, and Luminex (Table 1). For immunohistochemistry and morphological analysis, mice were deeply anesthetized with sodium pentobarbitone (ip) and perfused with PBS containing 0.1% heparin followed by 4% paraformaldehyde in Sorenson's phosphate buffer solution (pH 7.4). Brains were removed and postfixed overnight at 4 C in 4% paraformaldehyde and embedded in paraffin. Serial 10- $\mu$ m coronal sections were cut using a microtome and collected onto glass slides (Superfrost Plus; Menzel Glaser, Singapore), with each series consisting of 10 slides and each slide containing sections that were 200  $\mu$ m apart.

For protein analysis, mice were euthanized via an overdose of sodium pentobarbitone (ip). Brains were removed, snap frozen in liquid nitrogen, and stored at –80 C until further use. Brains

**TABLE 1.** Numbers and ages of mice in each experimental group

	Western blot/ELISA/Luminex		Immunohistochemistry	
	n	Average age (age range)	n	Average age (age range)
<i>hpg</i> male	6	15 months (12–17 months)	4	15 months (12–17 months)
Wild-type male	5	All 17 months	3	All 17 months
<i>hpg</i> female	6	18.5 months (17–19 months)	4	18.5 months (18–19 months)
Wild-type female	4	17.5 months (17–18 months)	2	18 months (17–19 months)

were divided into two hemispheres: one for Western blot and the other for ELISA/Luminex.

### Periodic-acid Schiff (PAS) staining and immunohistochemistry

Hippocampal morphology and glycogen-rich regions were observed in sections stained with the PAS stain and counterstained with hematoxylin. APP and GFAP protein localization was determined by fluorescent immunohistochemistry. Briefly, paraffin sections were dewaxed and rehydrated. Sections then underwent antigen retrieval by microwave boiling in 10 mM citrate buffer (pH 6) followed by successive washes in PBS, 80 mM NH<sub>4</sub>Cl (pH 7.4), and PBS for 10 min each. Sections were blocked with blocking buffer (PBS; 10% normal goat serum; 0.2% Triton-X100) for 1 h at room temperature and incubated overnight (4 C) with primary antibodies against APP (Rb194; 1:500) and GFAP (1:500) diluted in blocking buffer. Sections were washed with PBS, incubated with antimouse fluorescein isothiocyanate (1:100; ICN Cappel, Costa Mesa, CA) and antirabbit Cy3 (1:300; Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibodies diluted in blocking buffer for 2 h at room temperature, counterstained with Hoechst 33342 (Sigma; 4  $\mu$ l/ml in PBS), and coverslipped with Dako fluorescent mounting media (DAKO Corp., Carpinteria, CA). Images were collected using a multiphoton laser scanning confocal microscope.

Peroxidase immunohistochemistry for P450scc and ChAT was performed. Sections were dewaxed and rehydrated and underwent antigen retrieval using the method described above. Sections were blocked with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for P450scc immunohistochemistry and 2% H<sub>2</sub>O<sub>2</sub> in PBS for ChAT immunohistochemistry, each for 20 min at room temperature. Sections were incubated in blocking buffers individually optimized for P450scc (PBS; 5% normal goat serum; 3% skim milk; 0.5% Triton-X100) and ChAT (PBS; 10% horse serum; 0.2% Triton-X100) for 2 h and 1 h, respectively. Sections were incubated with primary antibodies against P450scc (1:100) and ChAT (1:200) overnight at 4 C. Sections were washed with PBS and incubated with biotinylated secondary antibodies (1:800; Vector Laboratories, Inc., Burlingame, CA) for 2 h at room temperature. Sections were washed with PBS and incubated with an avidin/horseradish peroxidase solution (Vectastain Elite ABC kit, diluted 1:100) for 1 h at room temperature. After further washes in PBS, sections were incubated in metal enhanced 3,3'-diaminobenzidine substrate (Pierce Chemical Co., Rockford, IL) and coverslipped with DPX mounting media.

### Western blot

Levels of APP, GFAP, apoE, PS1, and APP-CTF proteins were measured in the hippocampus, cerebellum, and the rest of the brain using Western blot. Individual brain regions were homogenized in buffer (PBS; 100 mM butylated hydroxytoluene; 100 mM EDTA), and total protein levels were determined. Samples were added to sodium dodecyl sulfate loading buffer (166 mM Tris-HCl, 8% sodium dodecyl sulfate, 4% glycine, 2.5% 2-mercaptoethanol, pinch of phenol red, pH 6.8), boiled at 95 C for 10 min and loaded onto Tris-trycine polyacrylamide gels. Proteins were separated using electrophoresis and transferred to nitrocellulose membranes at 250 mA overnight at 4 C. Membranes were blocked with 5% skim milk in Tris-buffered saline for 1 h at room temperature and immunoblotted for APP/APP-

CTF (C1/1.6; 1:5,000), GFAP (1:10,000), apoE (1:10,000), and PS1 (antibody 14; 1:2,000). Membranes were then incubated with antimouse (1:5,000; GE Healthcare, Piscataway, NJ), antigoat (1:10,000; Dako) or antirabbit (1:5,000; GE Healthcare) horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ) and developed onto film. Membranes were stripped (Restore plus Western blotting stripping buffer; Thermo Scientific, Rockford, IL) and probed for  $\beta$ -actin (1:10,000; Abcam, Cambridge, MA) to check for equal protein loading. Films were scanned using a visual light scanner, and intensity for each protein was analyzed using Quantity One 1-D Analysis Software (Bio-Rad Laboratories, Inc., Hercules, CA). All samples were performed in duplicate, and *hpg* protein levels were normalized to wild-type levels for statistical analysis.

### A $\beta$ ELISA

A $\beta$ 40 and A $\beta$ 42 levels were quantified in the whole-brain hemisphere using ELISA as previously described (34). ELISA plates were coated with the capture antibody 6E10 (1:400; Signet Diagnostic, Hicksville, NY), and detection antibodies against A $\beta$ 40 and A $\beta$ 42 were donated by Dr. Pankaj Mehta (New York State Institute for Basic Research). The optical density was measured at 450 nm using a FLUOstar OPTIMA multifunction microplate reader. All samples were run in duplicate.

Because other molecular changes were only observed in the hippocampus of male *hpg* mice, A $\beta$ 40 and A $\beta$ 42 levels were also quantified in the hippocampus of additional male *hpg* ( $n = 7$ ) and wild-type mice ( $n = 7$ ) aged 12 months. Hippocampal A $\beta$ 40 and A $\beta$ 42 levels were measured using high-sensitivity ELISA kits (Wako Chemicals USA, Richmond, VA) according to manufacturer's instructions, and all samples were run in duplicate.

### Luminex

The concentration of IL-1 $\beta$  and IL-6 protein in the brain was measured using Luminex. Protein was extracted from the whole-brain hemisphere using the same technique as for the A $\beta$  ELISA. The assay was performed according to the manufacturer's instructions for the Lincoplex kit (Linco Research, Inc., St. Charles, MO; catalog no. MICYTO-70K).

### Statistical analysis

Statistical analysis was performed using SPSS (Version 17). When directly comparing two groups, two-tailed independent  $t$  tests were performed. When comparing more than two groups, univariate ANOVA was performed. Levene's test was performed to test the equality of variance assumption for ANOVA. The intensity of ChAT immunostaining was ranked on an ordinal scale of 1–5, and the non-parametric Kruskal-Wallis test was used to perform statistics to determine whether there was a significant difference in the intensity of ChAT staining between *hpg* and wild-type mice.

## Results

### Hippocampal morphology

The morphology of the hippocampus was examined in PAS-stained sections and was found to be similar in *hpg*

and wild-type mice. Individual hippocampal subregions were qualitatively examined to determine whether specific hippocampal cell populations were affected in *hpg* mice. The area and width of the pyramidal cell layer of Ammon's horn (including CA1–3) and the granule cell layer of the dentate gyrus was examined using ImageJ (National Institutes of Health, Bethesda, MD) in three randomly selected coronal sections per brain ranging from  $-1.9$  to  $-2.3$  mm from Bregma, each  $200\ \mu\text{m}$  apart. There were no gross differences observed in the area or width of the pyramidal or granule cell layers between *hpg* and wild-type mice of either gender.

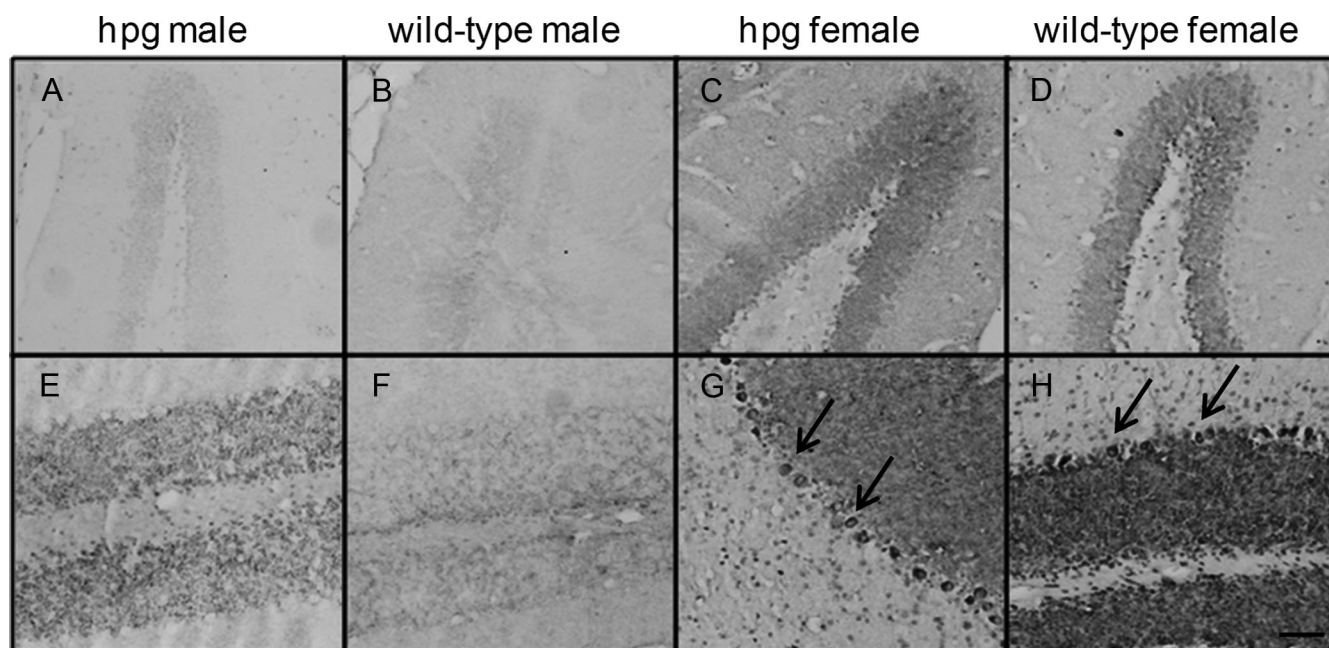
### Neurosteroidogenesis

Although it is well known that circulating sex steroid levels are low in *hpg* mice, neurosteroidogenesis has not been previously examined in *hpg* mouse brains. Therefore the level of P450scc, one of the rate-limiting factors for steroidogenesis (35), was examined in the caudal hippocampus and cerebellum, both regions previously shown to be associated with high levels of neurosteroidogenesis (36). Whereas female mice had increased P450scc staining compared with male mice in both brain regions, the level of P450scc staining was not consistently different between *hpg* and wild-type mice of either gender (Fig. 1). Females had more P450scc in all regions examined, including the hippocampus, cerebellum, medulla, and midbrain (Fig. 1). These gender differences were particularly obvious in the

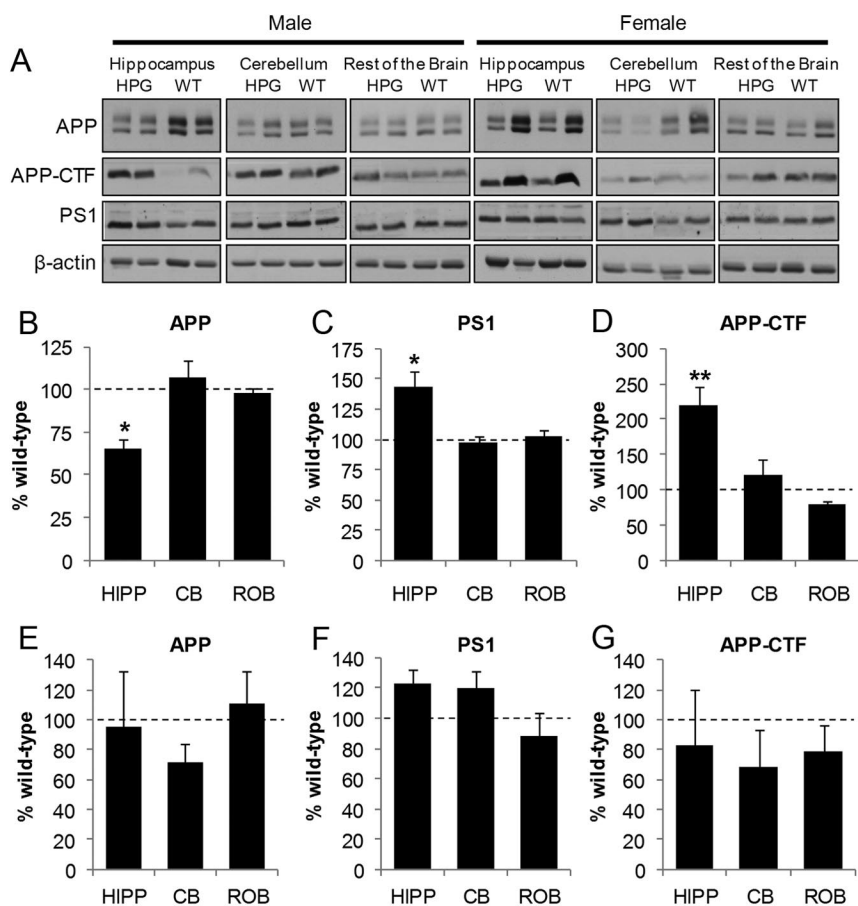
cerebellum, with a subset of Purkinje cells staining positive for P450scc in female brains, but not in male brains.

### Levels of proteins associated with $\beta$ -amyloid production

A consistent finding was that the brains of *hpg* male mice contained significantly different levels of proteins involved in amyloidogenic APP processing compared with male wild-type mice, including full-length APP, APP-CTF, and PS1, an integral component of  $\gamma$ -secretase. Male *hpg* mice had significantly lower levels of APP (*t* test;  $P < 0.05$ ) and significantly higher levels of APP-CTF (*t* test;  $P < 0.01$ ) and PS1 (*t* test;  $P < 0.05$ ) in the hippocampus than male wild-type mice (Fig. 2). In contrast, protein levels of APP, APP-CTF, and PS1 in the cerebellum and rest of the brain were not significantly different between male *hpg* and male wild-type mice. Immunohistochemistry was used to determine the cellular location of APP in the hippocampus. *Hpg* and wild-type mice had similar APP cellular localization, with immunopositive staining primarily observed throughout the soma of neurons in Ammon's horn regions and the dentate gyrus, similar to previous studies (37). A small proportion of immunopositive staining was also observed in astrocytes and in the nuclei of some cells (data not shown). Importantly, although there was some variation in the age of male *hpg* mice at the time of euthanasia (12–19 months), there was no relationship observed between age and levels of these AD-related proteins



**FIG. 1.** P450scc immunohistochemistry in the hippocampus (A–D) and cerebellum (E–H). P450scc expression was higher in the female brain than in the male brain in both brain regions, regardless of whether it was from *hpg* or wild-type mice. Expression was particularly high in the female cerebellum (G and H) with positive staining observed in Purkinje cells (arrows in G and H) that was absent in the male cerebellum (E and F). Scale bar,  $100\ \mu\text{m}$



**FIG. 2.** Western blot analysis of protein expression of APP, PS1, and APP-CTF in the hippocampus (HIPP), cerebellum (CB), and rest of the brain (ROB) in male and female *hpg* mice. Representative blots for each protein are shown in panel A. *Graphs* show average density of *hpg* protein bands normalized to wild-type (WT) levels, depicted by the *dashed line* at 100%. Male *hpg* protein levels shown in graphs B–D and female *hpg* protein levels shown in graphs E–G. The normalization to WT protein levels allows data to be presented as the average protein levels from two duplicated Western blots that were performed to ensure consistency.  $\beta$ -Actin was used as the loading control. Data are shown as mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  vs. WT protein level.

measured using either Western blot or immunohistochemistry; therefore all mice were included in the analysis.

Changes in the total level of APP, APP-CTF, and PS1 proteins in the male *hpg* hippocampus could suggest an increase in amyloidogenic processing. To test this, levels of  $A\beta_{40}$  and  $A\beta_{42}$  were initially measured in the entire brain hemisphere using ELISA. There was no difference in the amount of  $A\beta_{40}$  or  $A\beta_{42}$  between *hpg* and wild-type mice of either gender (ANOVA; Fig. 3, A and B). Because the differences in APP, APP-CTF, and PS1 protein levels were specifically observed in the hippocampus and not in other brain regions, it was hypothesized that increased  $A\beta$  levels may only be observed in the hippocampus. Therefore,  $A\beta_{40}$  and  $A\beta_{42}$  protein levels were measured specifically in the hippocampus of additional aged male *hpg* and wild-type mice. It was found that male *hpg* mice had significantly higher  $A\beta_{42}$  levels in the hippocampus than male wild-type mice ( $t$  test;  $P < 0.05$ ; Fig. 3D). Although male

*hpg* mice also had higher  $A\beta_{40}$  protein levels in the hippocampus than male wild-type mice, this difference was not significant ( $t$  test;  $P = 0.113$ ; Fig. 3C). These data therefore suggest that male *hpg* mice had increased amyloidogenic processing of APP that resulted in increased expression of  $A\beta_{42}$  specifically in the hippocampus and not in other brain regions.

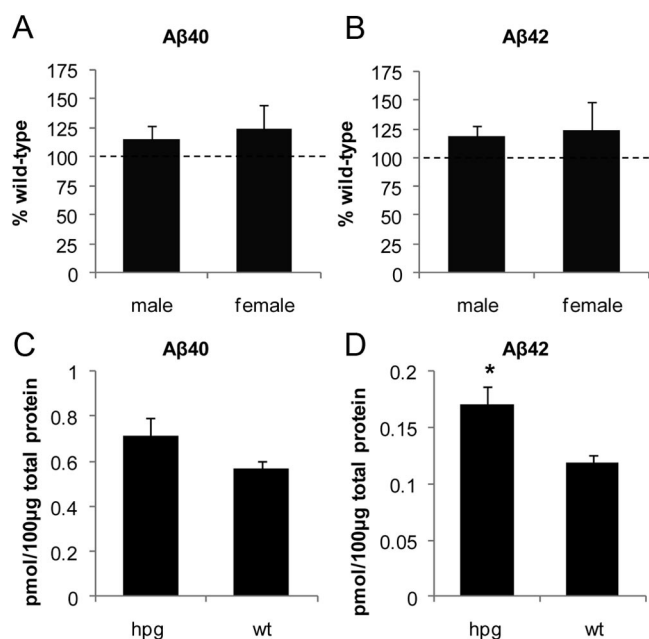
In contrast to male mice, there was no difference in the amount of proteins related to amyloidogenic processing of APP between female *hpg* and wild-type mice. Western blot analysis showed no significant difference in the amount of APP, APP-CTF, or PS1 in the hippocampus, cerebellum, or rest of the brain between female *hpg* and wild-type mice (Fig. 2). There was also no difference in the amount of  $A\beta_{40}$  or  $A\beta_{42}$  in the whole-brain hemisphere between female *hpg* and wild-type mice (Fig. 3, A and B).

Immunohistochemistry for APP revealed areas in all brains that were intensely stained for APP (Fig 4, A and B). These intensely stained areas had a whorl-like appearance and hence are referred to as “whorls” from here on. Interestingly, whorls were only observed in the thalamus and not in any other brain regions. When APP immunohistochemistry was costained with

GFAP and Hoechst 33342, it was found that whorls contained only APP, whereas GFAP-positive processes surrounded the central whorl. Whorls were also positive for PAS staining (Fig 4, C and D). PAS staining primarily stains glycogen-rich regions but has also been shown to stain amyloid deposits (38, 39). The total number of whorls was counted across three coronal sections per brain that were 200  $\mu$ m apart through the hippocampus. The size of each of the whorls was also calculated, and it was found that the number and size of the whorls were similar in all animal groups, regardless of gender or depletion of sex hormones.

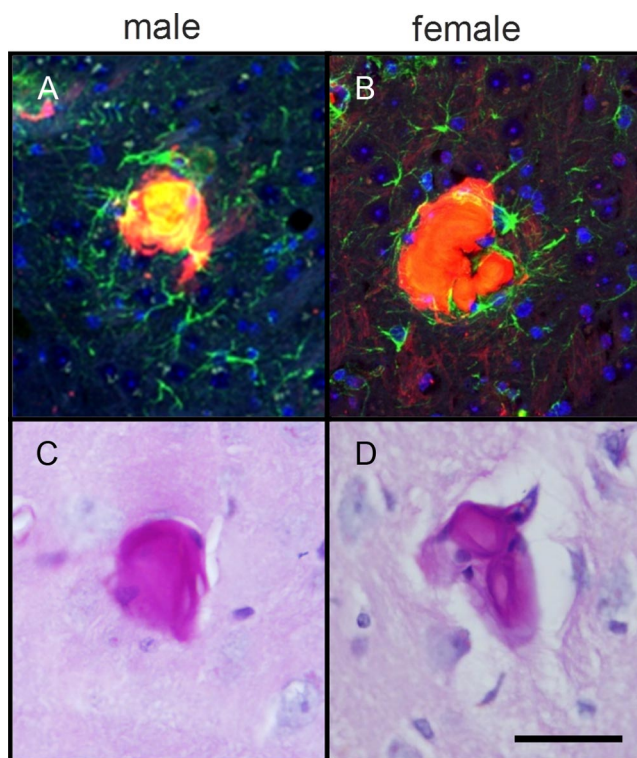
### ChAT protein

ChAT is an enzyme involved in the production of acetylcholine and is depleted in AD (40); thus ChAT expression in *hpg* mice was assessed using immunohistochemistry. In all brains, ChAT-positive cells were found within



**FIG. 3.** Aβ40 and Aβ42 levels in the whole-brain hemisphere (A and B) and hippocampus (C and D) of *hpg* mice measured using ELISA. Aβ levels in the whole-brain hemisphere were normalized to the wild-type (wt) male and female levels that are depicted as *dashed line* at 100%. Aβ levels in the hippocampus were only measured in male *hpg* and wt mice. Data are shown as mean ± SEM; \*,  $P < 0.05$

the caudate/putamen and in the basal forebrain (including the medial and lateral septal nuclei, substantia innominata, and the nucleus of the diagonal band of Broca). The total number of ChAT-positive cells was counted across a series of six 10-µm sections, each 300 µm apart per animal, between +1.24 mm and -0.65 mm from Bregma. Cells were divided into two groups: those in the caudate/putamen and those in the basal forebrain, and the total number of ChAT-positive cells was counted in each region (Fig. 5, E and F). The number of ChAT-positive cells was not affected in either region by gender or depletion of sex hormones. However, the amount of ChAT staining in each individual cell was qualitatively different in *hpg* and wild-type mice. The staining intensity of ChAT-positive cells was graded on an ordinal scale of 1–5 (1, faint immunostaining; 5, intense immunostaining) in five sections per animal. Although there was occasional variability of ChAT staining between sections from each animal, overall it was found that ChAT staining was less intense in sections from male *hpg* mice in comparison with those from male wild-type mice (Kruskal-Wallis;  $P < 0.05$ ), and there was no apparent difference in the amount of ChAT staining between female *hpg* and wild-type mice. Representative images of ChAT intracellular staining intensity are shown in Fig. 5. There was also a gender difference in the staining pattern in ChAT-positive cells regardless of sex hormone depletion; male ChAT positive cells had a



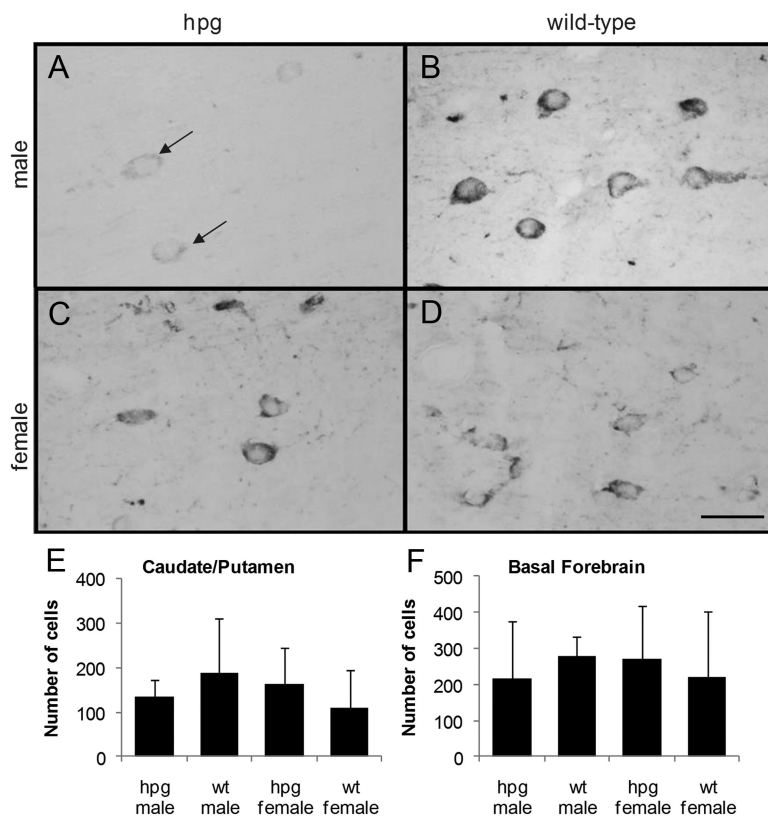
**FIG. 4.** APP immunopositive deposits in male (A and C) and female (B and D) *hpg* brains. A and B show concentrated areas of APP immunopositive staining (red) that were surrounded by GFAP immunopositive astrocytes (green). Sections were also counterstained with Hoechst to label nuclei (blue). These deposits were also PAS stain positive (C and D). Scale bar, 50 µm

rounder shape and more consistent staining throughout the cytoplasm, whereas female cells were smaller and less round, and cytoplasmic staining was more granular.

### Inflammatory and other changes

There was no significant difference in the amount of GFAP protein between male *hpg* and wild-type mice in any brain region measured using Western blot (data not shown). In contrast, female *hpg* mice had significantly lower total GFAP protein in the cerebellum than female wild-type mice ( $t$  test;  $P < 0.01$ ), but this effect was not observed in the hippocampus or rest of the brain. GFAP expression was also examined in the hippocampus using immunohistochemistry, but there were no consistently observable differences between *hpg* and wild-type mice or between male and female mice. ApoE protein levels were also examined using Western blot but there was no significant difference in the amount of apoE between *hpg* and wild-type mice in any brain region, in either gender (data not shown).

Inflammation is a key pathological feature of AD (for review see Ref. 41). To test whether *hpg* mouse brains were in a heightened inflammatory state, IL-1β and IL-6 were measured in the whole-brain hemisphere using Lu-

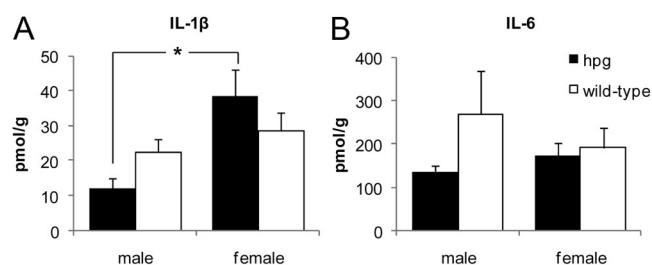


**FIG. 5.** Immunohistochemistry for ChAT in the basal forebrain (A–D). ChAT immunostaining per neuron was less intense in male *hpg* mice (A) than in male wild-type (wt) mice (B). A similar effect was not observed in female brain (C and D). The total number of ChAT-positive neurons was counted in the caudate/putamen (E) and in the basal forebrain (F). The total number of ChAT-positive neurons in *hpg* mice was not significantly different from wt mice of either gender. Data are shown as mean  $\pm$  SD; scale bar, 50  $\mu$ m

minex. There was a strong trend for brains from male *hpg* mice to have less IL-1 $\beta$  protein than brains from male wild-type mice (*t* test;  $P = 0.057$ ; Fig. 6A). Male *hpg* mice also had less IL-6 on average, but this was not statistically significant due to high interanimal variation in the male wild-type animals (Fig. 6B). Again, there was no difference in levels of either IL-1 $\beta$  or IL-6 between the female *hpg* and wild-type mice.

## Discussion

The lifelong lack of sex hormones resulted in gender-specific changes, with male *hpg* mice exhibiting a number of



**FIG. 6.** The concentration of IL-1 $\beta$  (A) and IL-6 (B) in *hpg* and wild-type mouse brains. Cytokine levels measured using Luminex in the whole-brain hemisphere. Data are shown as mean  $\pm$  SEM; \*,  $P < 0.05$ .

AD-like changes including altered levels of APP, APP-CTF, PS1, and A $\beta$ 42 proteins in the hippocampus, decreased ChAT immunoreactivity per neuron in the basal forebrain, and a trend for lower IL-1 $\beta$  levels. In contrast, the only significant difference observed between female *hpg* and wild-type mice was that female *hpg* mice had significantly less GFAP in the cerebellum than wild-type mice.

It is interesting that AD-related changes were only observed in male, but not female *hpg* mice, considering that a number of previous papers have observed an increase in A $\beta$  production and other AD-related changes after ovariectomy (21, 23, 42). One explanation as to why AD-related changes were only observed in male *hpg* mice is that female mice may have an inherently higher level of neurosteroidogenesis, as suggested by the higher P450scc levels in female mice shown in this study. In addition to sex steroids in the blood, sex steroids in the brain may also cause functional changes; thus future research is needed to determine whether there is a gender difference in

other enzymes involved in neurosteroidogenesis in *hpg* mice and hence in brain levels of sex steroids. An alternative or additional explanation is that the AD-associated changes in male *hpg* mice were mediated by androgens. The increased amount of A $\beta$ 42 observed in the hippocampus of male *hpg* mice supports previous studies that have shown that androgen depletion by GDX of male animals increases A $\beta$ , and supplementation with exogenous androgens decreases A $\beta$  levels (17–19, 43–45). The underlying mechanism of how androgens modulate A $\beta$  levels is still under investigation, but it could be via altering the level of amyloidogenic processing of APP or by altering degradation of A $\beta$ .

The decreased APP protein, coupled with increased APP-CTF and PS1 proteins in the hippocampus of male *hpg* mice, suggests that depletion of androgens promotes increased amyloidogenic processing of APP. It is hypothesized that the decreased levels of APP is a consequence of increased cleavage of APP by BACE into APP-CTF and that PS1 expression is up-regulated to cleave the higher amount of APP-CTF into A $\beta$ . This hypothesis is supported by a recent study that found increased testosterone expression due to knockout of aromatase resulted in de-



creased APP-CTF and APP intracellular C-terminal domain levels, coupled with decreased A $\beta$ 42:A $\beta$ 40 ratio. The altered protein levels were attributed to a testosterone-mediated decrease in amyloidogenic processing, primarily via decreased BACE expression and activity (44). Although BACE expression was not measured in the current study, results from McAllister *et al.* correlate well with the other protein changes observed in this study. Furthermore, testosterone supplementation after GDX results in decreased PS1 expression in adult mice (46), supporting the results from the current study and suggesting that androgen depletion may also affect expression of  $\gamma$ -secretase in addition to BACE.

It is important to note that some studies have been unable to confirm a similar relationship between androgen depletion and APP or APP-CTF levels (17–19) and have therefore concluded that androgen depletion does not affect amyloidogenic processing. The findings presented in this study likely differ from previous results because of the different hormonal profiles of the mice examined. Previous studies have used GDX to deplete androgens, which also results in increased blood levels of LH and FSH. In contrast, *hpg* mice have comparably low levels of circulating sex hormones (31) but also low levels of LH and FSH (30). This may be important because LH is also capable of promoting amyloidogenic processing and A $\beta$  production (47, 48). Given this capability, it is possible that the depletion of LH in male *hpg* mice was responsible for the altered levels of proteins related to AD rather than the depletion of androgens. However, if this were the case, one would expect male *hpg* mice to be protected against AD-associated changes and for male and female *hpg* mice to show the same phenotype because both sexes had similarly low LH levels. Therefore, it appears more likely that depleted levels of androgens were responsible for the AD-associated changes in male *hpg* mice.

The majority of previous studies have not examined protein effects that result from androgen depletion in individual brain regions. Because increased amyloidogenic processing only occurred in the hippocampus of male *hpg* mice, pooling of multiple brain regions would dilute this effect. Indeed, this was shown to be the case in the current study because A $\beta$ 40 and A $\beta$ 42 levels in *hpg* and wild-type mice were not significantly different when levels were measured in the complete brain hemisphere but were significantly different when levels were examined specifically in the hippocampus. It is interesting that the androgen-mediated changes in levels of proteins associated with AD were observed only in the hippocampus and not in other brain regions. Why the hippocampus is particularly vulnerable in AD is still a matter of debate, but the current results suggest that this may be at least partially mediated

by sex hormones. Estrogen receptor  $\alpha$ , estrogen receptor  $\beta$ , androgen receptors, and LH receptors are expressed in the hippocampus (49–51), suggesting that the hippocampus may be highly responsive to sex hormone-mediated effects. It has been suggested that sex hormones have an important role in mediating many features of the hippocampus, including hippocampal-dependent cognitive tasks, synaptic plasticity, and neurogenesis (for review see Ref. 52). Although only a few studies have compared the sex hormone-mediated effects in the hippocampus with other brain regions, it was recently shown that ovariectomy of female rats resulted in decreased expression of an A $\beta$ -degrading enzyme, insulin-degrading enzyme, specifically in the hippocampus and not in the cerebellum (42), hence supporting the current results showing that sex hormone-mediated effects on proteins related to AD are prominent in the hippocampus and not in other brain regions.

The presence of whorls in the thalamus of all aged mice, regardless of genotype, is interesting, especially considering that the primary component of these whorls was either full-length APP or a cleavage product of APP. It may be premature to describe these structures as amyloid plaques because rodent A $\beta$  has a different sequence than human A $\beta$ , and consequently does not generally aggregate and form amyloid plaques (53). However, it is important to note that rodent A $\beta$  has been shown to form plaque-like structures in other animal models (54, 55). The whorls observed in this study shared similar characteristics with amyloid plaques; they were composed of aggregated proteins that are immunopositive for both APP and PAS staining, and GFAP-positive processes circled around the whorls, with some processes entering the whorl itself. It is not yet known what these whorls are, or why they were observed specifically in the thalamus and not elsewhere in the aged mouse brain. Sex hormones did not appear to affect the number or size of these whorls, suggesting that this may be a feature of the C3H/J strain or a consequence of age.

Semiquantitative immunohistochemical analysis suggested a decrease in ChAT staining per neuron in the basal forebrain of male *hpg* mice compared with wild-type male mice. This finding supports previous studies that have shown GDX results in decreased ChAT activity in the male rodent brain (56, 57). Furthermore, GDX adult male rats have lower ChAT intensity per ChAT-positive cell than sham GDX animals or GDX animals supplemented with testosterone, similar to what was observed in this study (58). Interestingly, it has also been shown that testosterone depletion does not affect the number of ChAT-positive cells (59), only the ChAT staining intensity within cells, suggesting that testosterone depletion does not in-

duce cell death of ChAT-positive cells but instead down-regulates ChAT production within neurons. It is still unclear how androgens do this, but it is unlikely to be via an androgen receptor-mediated pathway because only 0.7–1.1% of cells in the basal forebrain contain both androgen receptors and ChAT (58). Therefore, the decreased expression of ChAT observed in male *hpg* mice was likely to be due to androgen-mediated effects that were not regulated by androgen receptors. Future studies that quantitatively measure ChAT expression and ChAT enzyme activity are needed to confirm the decreased ChAT staining in *hpg* male mice observed in this initial study and how these changes affect acetylcholine levels and cognitive function in aged *hpg* mice.

Markers of inflammation were examined in the brains of *hpg* mice to see whether circulating sex hormone depletion increased neuroinflammation, as has been suggested to occur in some (60–63), but not all (64–66), previous studies. Unexpectedly, male *hpg* mice had a strong trend for lower levels of IL-1 $\beta$  in the whole-brain hemisphere compared with wild-type mice, whereas there was no difference observed between female *hpg* and female wild-type mice. There was also no difference in the amount of GFAP protein in male *hpg* and wild-type brains in any brain region. Significantly lower GFAP protein levels were observed in the cerebellum of female *hpg* mice than in wild-type mice but not in any other region. These results do not support those by McQueen *et al.* (67) who found higher GFAP protein levels in the CA1 region in the hippocampus of male and female *hpg* mice, in comparison with wild-type mice. However, it is important to note that the earlier study semiquantified GFAP protein expression by counting the number of immunopositive cells only in CA1, rather than determining the total GFAP protein level in the whole hippocampus as measured in the current study. The fact that GFAP protein levels were only significantly different in the female cerebellum may suggest that GFAP is primarily regulated by estrogens rather than by androgens. The current data also do not support a relationship between sex hormones and apoE levels in males or females because there was no difference in apoE protein levels between *hpg* and wild-type mice of either gender. This result supports a recent study that found GDX and subsequent administration of exogenous estradiol or testosterone did not affect apoE mRNA or protein levels in the mouse brain (68). Overall these results suggest that the low levels of circulating sex hormones and gonadotropins in *hpg* mice do not increase brain inflammation, which in turn is not a cause or effect of the increased amyloidogenic processing observed in the male *hpg* hippocampus.

In conclusion, this study provides evidence for a link between the depletion of androgens and changes in levels

of proteins related to AD, specifically in the male hippocampus. This study has also revealed that *hpg* mice are a novel and potentially useful model for examining the relationship between long-term sex hormone depletion and brain changes associated with AD. Although it was beyond the scope of this study to definitively determine which individual sex hormone(s) mediated the AD-associated changes observed in male *hpg* mice and the underlying mechanism of androgen-mediated effects, these promising findings warrant future studies using *hpg* mice to examine pathology associated with AD.

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