Aging is associated with changes in allopregnanolone concentrations in brain, endocrine glands and serum in male rats

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Abstract

Objective: Allopregnanolone is a potent neuroactive steroid hormone produced in the brain and in peripheral endocrine glands. The present study investigated possible age-related variations in allopregnanolone content in brain areas, endocrine glands and serum of male rats.

Design: Wistar male rats were categorized into 5 groups (6 rats in each) according to age: 6, 12, 16, 18 and 20 months respectively.

Methods: Allopregnanolone content in acidic homogenates of brain cortex, hypothalamus, pituitary, adrenals and gonads was measured by a specific radioimmunoassay. Serum allopregnanolone, corticosterone and testosterone were also assayed by radioimmunoassay.

Results: Brain cortex allopregnanolone content decreased significantly with age, while hypothalamic allopregnanolone content remained constant until 18 months and increased significantly at 20 months. Pituitary content showed a significant age-related reduction. Adrenal allopregnanolone content remained constant until 18 months, and was significantly higher at 20 months. Testis and serum allopregnanolone contents showed significant age-related increases. Serum testosterone levels showed an age-related decrease, while no age-related variation in serum corticosterone was found.

Conclusions: The present study showed a significant impact of aging on allopregnanolone contents in brain, endocrine glands and serum, showing an age-related decrease in brain cortex and pituitary, and an age-related increase in testes, adrenals and serum.

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Introduction

Neuroactive steroids are substances synthesized in the brain, either \textit{de novo} from cholesterol or by \textit{in situ} metabolism of blood-borne precursors. Chemically characterized in other tissues but also synthesized within the brain, neurosteroids participate in the regulation of brain function (1–3). Among them, 3\textalpha\textbeta-hydroxy-5\textalpha-pregn-20-one (allopregnanolone) is one of the best represented and one of the most potent. Allopregnanolone may act as an endogenous ligand of GABA-\textalpha receptors and has anxiolytic-sedative effects in rats under physiological conditions such as stress or pregnancy (4, 5). An ovarian contribution to plasma and brain allopregnanolone concentration is indicated by the higher levels observed in female rats compared with age-related males and by the decrease in plasma levels after ovariectomy (6). An adrenal origin of allopregnanolone in both male and female rats has also been shown (6).

A correlation between allopregnanolone and reproductive life has been suggested. Rat hypothalamic and hippocampal contents of allopregnanolone show significant changes during the estrous cycle (7, 8). No data on brain allopregnanolone content are available in aged rats. The observation that allopregnanolone modulation of the stress response is impaired in aged rats (8) and that the plasma levels of other neuroactive steroids, such as dehydroepiandrosterone (DHEA) undergo changes with aging in rats and in man (9–11), led us to investigate possible age-related variations in allopregnanolone levels in different brain areas, endocrine glands, and serum of male rats.

Materials and methods

Wistar male rats (5 groups of 6 rats each) were grouped according to their age: 6, 12, 16, 18 and 20 months respectively. All rats were kept in the laboratory for at least a week before being killed; they were exposed to 12 h light/day and food and water were available \textit{ad libitum}. Animals were killed by decapitation.
Brain cortex, hypothalamus, pituitary, adrenals and testes were quickly removed, weighed and homogenized in ice-cold 50% aqueous methanol containing 1% acetic acid with 3000 d.p.m. tritium-labeled steroid as internal recovery standard. The homogenate was centrifuged at 1200 r.p.m. for 15 min at 4°C. Blood was collected in plastic tubes, centrifuged at 3500 r.p.m. for 10 min, and the serum was stored at −20°C until assay. The supernatants of the tissue homogenates and serum were passed through a C-18 Sep-Pak cartridge, previously equilibrated with the homogenizing buffer. The cartridge was sequentially washed with homogenizing buffer, 50% aqueous methanol, and the unconjugated steroid fraction was eluted with absolute methanol and brought to dryness under nitrogen. Analytical grade solvents were purchased from Merck (Darmstadt, Germany); C-18 Sep-Pak cartridges were obtained from Waters Corporation (Milford, MA, USA). Allopregnanolone contents were measured by radioimmunoassay (RIA). Standard allopregnanolone was purchased from Sigma Chemical Co. (St Louis, MO, USA) and pregnan-3α-ol-20-one, 5α-[9,11,12-3H(N)] (45 Ci/mmol) was from Amersham (Little Chalfont, Bucks, UK). Polyclonal antisera raised in sheep against allopregnanolone carboxymethyl ether coupled to bovine serum albumin (kindly provided by Dr Purdy) have been employed.

For further validation of the assay procedure, we used an internal recovery standard of tritium-labeled allopregnanolone for each sample; data were corrected for procedural loss. Serum samples containing an internal recovery standard of [3H]allopregnanolone were extracted according to the same procedure. The antiserum was employed at a final working dilution of 1:4000, determined from its ability to bind 50 pg labeled steroid. The cross-reactivity of the sheep antisera against 3α-hydroxy-20-oxo-5α-pregnan-11α-yl carboxymethyl ether coupled to bovine serum albumin was determined by testing the ability of various structurally similar compounds to displace tritiated allopregnanolone from the antibody. The only significant cross-reactivity occurred with 3α-hydroxy-5β-pregnan-20-one (Table 1), which is not found in appreciable concentration in rat tissues where 5α-reductase activity predominates.

The sensitivity of the assay was 15 pg/ml and the losses during sample processing were determined as recovery of the tracer (1500 d.p.m. [3H]allopregnanolone) added to analyzed serum; the average recovery was 85.5 ± 5.7% (mean ± S.E.M.) and the intra- and interassay coefficients of variation were 7% and 9% respectively.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Cross-reactivities of the sheep antisera against 3α-hydroxy-20-oxo-5α-pregnan-11α-yl carboxymethyl ether coupled to bovine serum albumin.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid</td>
<td>Cross-reactivity (%)</td>
</tr>
<tr>
<td>3α-Hydroxy-5α-pregnan-20-one</td>
<td>100.00</td>
</tr>
<tr>
<td>Pregnen-4-ene-3,20-dione</td>
<td>0.70</td>
</tr>
<tr>
<td>5α-Pregnan-3,20-dione</td>
<td>0.10</td>
</tr>
<tr>
<td>5β-Pregnan-3,20-dione</td>
<td>0.10</td>
</tr>
<tr>
<td>3α-Hydroxy-5β-pregnan-20-one</td>
<td>6.50</td>
</tr>
<tr>
<td>20β-Hydroxy-5α-pregnan-3-one</td>
<td>0.10</td>
</tr>
<tr>
<td>3β-Hydroxy-5α-pregnan-20-one</td>
<td>0.05</td>
</tr>
<tr>
<td>3β-Hydroxy-5β-pregnan-20-one</td>
<td>0.01</td>
</tr>
<tr>
<td>5α-Pregnan-3α,20α-diol</td>
<td>0.10</td>
</tr>
<tr>
<td>5α-Pregnan-3β,20α-diol</td>
<td>0.01</td>
</tr>
<tr>
<td>5β-Pregnan-3α,20α-diol</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The following steroids have a cross-reactivity <0.001%: 3α-hydroxy-5α, 17α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 17α-hydroxy-4-pregnan-3,20-dione, androstan-17-one, dehydroepiandrosterone, testosterone, 11β-hydroxytestosterone, cortisol, corticosterone, estrone, estradiol, estril.

Results

Brain cortex allopregnanolone content showed a significant progressive decrease with age (6.30 ± 0.32 ng/g at 6 months vs 3.00 ± 0.20 ng/g at 20 months; P < 0.001 at 16, 18 and 20 months vs 6 months), while hypothalamic allopregnanolone content was quite constant until 18 months and increased significantly at 20 months (1.95 ± 0.05 ng/g; P < 0.01 vs 6 months) (Fig. 1). Pituitary allopregnanolone showed a statistically age-related significant reduction (2.98 ± 0.12 ng/g at 6 months vs 0.75 ± 0.03 ng/g at 20 months; P < 0.001 at 12, 16, 18 and 20 months vs 6 months) (Fig. 2). Adrenals allopregnanolone content in the adrenal gland was quite constant until 18 months, and increased significantly at 20 months (52.9 ± 0.3 ng/g; P < 0.01 vs 6 months) (Fig. 2). The content of allopregnanolone in the testis showed a clear age-related variation, increasing gradually from 0.062 ± 0.007 ng/g at 6 months to 0.35 ± 0.03 ng/g at 20 months (P < 0.001 at 16, 18 and 20 months vs 6 months) (Fig. 2).
Serum allopregnanolone was detectable in all rats and significantly and progressively increased with age (from $0.23 \pm 0.05$ ng/ml at 6 months to $0.31 \pm 0.03$ ng/ml at 18 months and $0.42 \pm 0.06$ ng/ml at 20 months; $P < 0.001$). No significant age-related variation in serum corticosterone was found. On the other hand, serum testosterone levels showed a significant age-related progressive decrease (from $2.00 \pm 0.08$ ng/ml at 6 months to $0.81 \pm 0.06$ ng/ml at 20 months; $P < 0.001$ at 16, 18 and 20 months vs 6 months) (Fig. 3).

**Figure 1** Mean ± S.E.M. age-related decrease in allopregnanolone content in the cortex ($y = -107.224x + 2955.193$, $r^2 = 0.778$, *$P < 0.001$ vs 6 months) and increase in allopregnanolone content in the hypothalamus at 20 months (*$P < 0.01$ vs 6 months) in male rats.

**Figure 2** Mean ± S.E.M. age-related changes in allopregnanolone content in the pituitary ($y = -38.372x + 977.961$, $r^2 = 0.906$, *$P < 0.001$ vs 6 months), adrenal ($y = 44.942x + 667.974$, $r^2 = 0.151$, *$P < 0.01$ vs 6 months) and testis ($y = 12.459x - 59.72$, $r^2 = 0.605$, *$P < 0.001$ vs 6 months) in male rats.
Discussion

The present study is the first to describe age-related changes in allopregnanolone content in various cerebral areas and endocrine glands in male rats. Data concerning allopregnanolone levels in some rat brain areas according to stress and the stage of the estrous cycle have been reported previously (8, 12, 13) but allopregnanolone variations with age have never been described.

In this study, allopregnanolone evaluation was performed by radioimmunological assay. It has been reported that radioimmunological methods are not sufficiently specific for assessing neurosteroid content, and that high performance liquid chromatography coupled with gas chromatography-mass fragmentography is a more reliable method for these studies (14). However, in the present study, the use of new more specific antisera appears to be a satisfactory alternative to the latter method, which is unsuitable for routine allopregnanolone assessment.

The present data show a significant decrease in brain cortex allopregnanolone content starting at 16 months of age, while an increase in hypothalamic allopregnanolone content at 20 months is observed. Allopregnanolone contents in brain cortex and hypothalamus of adult male rats are similar to those described in the literature (12, 13). The present data and the increase in cerebral allopregnanolone content under stressful conditions suggest that the stress-induced increase and the age-related decrease in brain allopregnanolone may have important behavioral and/or neuroendocrine consequences (12, 13).

It has been hypothesized that the stress-induced increase in allopregnanolone results in a diminished release of corticotropin releasing factor and adrenocorticotropic hormone (ACTH)/corticosterone, determining a novel feedback loop which decreases the activity of the hypothalamus-pituitary-adrenal axis under stress (12). The age-related decrease of hypothalamus-pituitary-adrenal activity in response to stress may occur because of the age-related decrease in cerebral allopregnanolone content.

A significant reduction in hypothalamic allopregnanolone content at proestrus also suggests a putative role of neurosteroids in the modulation of the ovulatory process (8).

It has been demonstrated that the anterior pituitary of female rats metabolizes progesterone to allopregnanolone and that this process is altered during reproductive senescence in female rats (15). No data on age-related variations in allopregnanolone pituitary content in male rats are available and the present study shows that pituitary allopregnanolone concentration in male rats significantly decreases from 6 months to 20 months of age.

The 5α-reductase-3α-hydroxysteroid-dehydrogenase system is responsible for the conversion of progesterone into allopregnanolone in rat brain and adrenal cortex.
into 5a-pregnan-3α-ol-20-one and then into 5a-pregnan-3α-ol-20-one. Rat adrenal glands express a 5α-steroid reductase and produce allopregnanolone in quantities similar to those secreted by the rat ovary (16). The results of the present study concerning adrenal allopregnanolone content in adult male rats are consistent with previous data (6), showing a significant increase in allopregnanolone adrenal content in aged rats.

The synthesis of pregnane compounds in the rat female ovary has been clearly demonstrated (17), while previous studies considered allopregnanolone undetectable in the tests (6). In contrast, the specific radioimmunoassay used in the present study was able to detect allopregnanolone in the tests, showing an increasing concentration from 6 to 20 months of age. Serum allopregnanolone levels paralleled the age-related increase in adrenal and testis allopregnanolone contents. These findings support the hypothesis that the adrenals and testes may be the main sources of circulating allopregnanolone in male rats (6).

In the present study no significant age-related variations in serum corticosterone were found, while a significant age-related progressive decrease in testosterone was detected, thus confirming previously reported data (18, 19). It has been hypothesized that an increase in adrenal sensitivity to ACTH is responsible for the maintenance of corticosterone levels in the presence of diminished ACTH levels in aged rats (18).

The opposite variations in allopregnanolone contents in brain cortex and serum suggest that the concentration of allopregnanolone in the brain is independent of circulating allopregnanolone. It has been suggested that the main site of formation of cerebral allopregnanolone is the brain itself, although serum progesterone constitutes the major precursor for allopregnanolone synthesis in the brain (6, 12). The higher cerebral allopregnanolone content observed in young rats may be a consequence of higher levels of 5α-reductase activity. In situ hybridization studies have shown that 5α-reductase mRNA is highly expressed in the pre- and early postnatal rat central nervous system, while it is expressed at low levels rather uniformly in the adult rat brain (20). No data are available on enzyme activity in aged rats.

The discussion remains open on the possible target of brain allopregnanolone. The increased synthesis of allopregnanolone in rat brain following acute stress suggested that this steroid may play a role as an endogenous stress-protective compound (21). Pretreatment with high doses of allopregnanolone significantly decreased corticotropin-releasing hormone-induced behavioral manifestations of stress and anxiety, indicating an anxiolytic, sedative-hypnotic and anti-aggressive effect similar to that produced by the benzodiazepines (22). A putative inhibitory role of allopregnanolone on the response of corticosterone to stress has been hypothesized. In fact, it has been reported that in aged rats treatment with antiserum raised against allopregnanolone failed to modify serum corticosterone levels following acute physical stress, as it does in adult rats (23). Therefore, it is possible that with advancing age neurosteroids lose part of their activity in the control of the hypothalamus–pituitary–adrenal axis (11). The significant age-related decrease in brain and pituitary allopregnanolone concentration observed in the present study supports this hypothesis.

In addition, the age-related decrease in allopregnanolone concentration in the brain may have an impact on stress, memory, anxiety and sexual behavior. These functions, typically altered in aged rats, may correspond to a variety of nervous and affective disorders observed in elderly men.

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References

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