Delayed testicular aging in pituitary adenylate cyclase-activating peptide (PACAP) null mice

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Age-related decline in male sex hormones is a direct consequence of testicular aging. These changes in the hormonal complement cause physiological disturbances affecting the quality of life for millions of aging men. To assess the influence on testicular aging of pituitary adenylate cyclase-activating peptide (PACAP), a polypeptide that regulates testicular steroidogenesis *in vitro*, we compared the testicular structure and function between C57BL/6 wild-type and PACAP$^{-/-}$ male mice, at 4 and 15 months of age. We show that, in 4-month-old PACAP$^{-/-}$ mice, steroidogenesis (evaluated by levels of testosterone, steroidogenic acute regulatory protein, 3β-hydroxysteroid dehydrogenase, and P450c17) was impaired. However, the testicular structure of these animals was not affected. At 15 months of age, wild-type testis displayed typical signs of aging (patchy seminiferous tubules, germ cell depletion, and vacuolization), whereas testicular structure was remarkably well conserved in PACAP$^{-/-}$ animals. The depletion of germ cells found in wild-type animals was associated with a higher content of peroxynitrites, a marker of reactive oxygen species, and a higher number of apoptotic cells compared with PACAP$^{-/-}$ mice. Our results show that testicular aging is delayed in PACAP$^{-/-}$ animals. Because the expression levels of steroidogenic factors are low and constant over time in knockout animals, a proposed mechanism for the protection against testicular degeneration is that production of reactive oxygen species, a byproduct of steroidogenesis that induces apoptosis, is down-regulated in PACAP$^{-/-}$ animals.

Leydig cells | reactive oxygen species | steroidogenesis | neuropeptide | andropause

The decline in male sex hormones affects a large proportion of the aging population. Approximately 30% of men >60 years of age have low serum testosterone levels (1) that most likely reflect a testicular aging process. Although the biological consequences of low testosterone have been well established (2, 3) (i.e., osteoporosis, reduced muscle strength, reduced libido, and mood changes), the mechanisms by which testosterone concentration declines with age are poorly understood. Reactive oxygen species (ROS) have been implicated as major factors in oxidative stress affecting a wide variety of physiological and pathological processes, including aging (4). Because steroidogenesis and, particularly, P450s activity is known to generate ROS (5, 6), it has been suggested that these local byproducts might be responsible for both functional deficits of Leydig cells during aging (5, 6) and germ cell apoptosis (7).

In animals, such as the Brown Norway rat, aging studies have linked the decline of testosterone production with a decrease in Leydig cell function, without reduction in Leydig cell number (8). Maintenance of Leydig cell function depends on pulsatile stimulation by the pituitary-produced luteinizing hormone (LH) (9). In both rats and humans, it has been shown that, during aging, although serum LH levels remain constant (8, 10), the reduction of steroidogenesis is partially attributable to alteration in LH pulsatility (11). However, exogenous administration of LH in a pulsatile fashion fails to rescue production of testosterone in Leydig cells in both rats and humans (12, 13). Furthermore, levels of testosterone produced by aging Leydig cells can be restored to young levels after administration of daily doses of testosterone over an extended period to suppress the hypothalamic–pituitary–gonadal axis (14). This effect suggests that long-term inhibition of pituitary LH production (induced by testosterone itself or other feedback mechanisms) may be sufficient to rejuvenate endogenous testosterone synthesis in aging Leydig cells. Taken together, these observations suggest that alteration in LH stimulation is not the sole factor explaining why Leydig cells become hypofunctional during aging.

Pituitary adenylate cyclase-activating peptide (PACAP), a member of the VIP neuropeptide family, is expressed in the testis (15). Because PACAP is a well known endogenous stimulator of cAMP production in many cellular systems (16), and cAMP is a key compound in activation of steroidogenesis (17), this peptide is a good candidate for a regulator of Leydig cell function. Moreover, PACAP has been shown to regulate pituitary LH secretion (18). PACAP and its mRNA, detected by immunohistochemistry and *in situ* hybridization in testes, is expressed in the germ cell lineage, including spermatogonia, primary spermatocytes, and round spermatids, but is not expressed in Sertoli or Leydig cells (19), suggesting that germ cell PACAP may act in a paracrine fashion on PAC1 receptor-expressing Leydig cells. Indeed, in cultured Leydig cells from both adult and fetal rat, PACAP induces stimulation of cAMP and testosterone secretion (20–22), showing that, *in vitro*, it exerts a direct effect on maturation and function of these cells. Furthermore, PACAP stimulates primordial germ cell proliferation *in vitro* (23) and induces the secretion of lactate and inhibin by Sertoli cells (24).

In this study, we show that young adult mice lacking PACAP display decreased steroidogenesis. In addition, testicular aging is delayed in these knockout (KO) animals. We propose that PACAP normally stimulates steroidogenesis in Leydig cells and, therefore, production of ROS. In turn, these toxic molecules would cause testicular structure degeneration, ultimately leading to testicular aging.

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Abbreviations: 3β-HSD, 3β-hydroxysteroid dehydrogenase; FSH, follicle-stimulating hormone; KO, knockout; LH, luteinizing hormone; PACAP, pituitary adenylate cyclase-activating peptide; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein.

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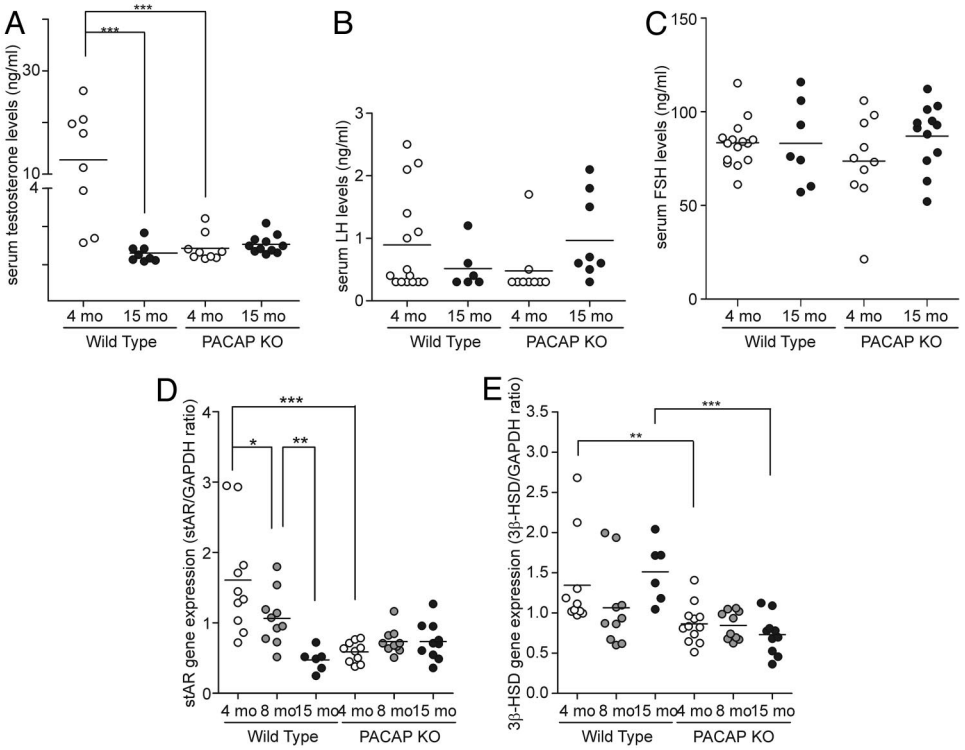

Fig. 1. Comparison of testosterone and steroidogenesis levels during aging between wild-type and PACAP$^{-/-}$ animals. (*A*) Serum testosterone levels during aging in wild-type and PACAP$^{-/-}$ males. Between 4 ($n = 8$) and 15 ($n = 8$) months of age, serum testosterone decreased dramatically in wild-type animals, whereas levels remain low and constant in PACAP$^{-/-}$ mice ($n = 9$ for 4-month-old and $n = 15$ for 15-month-old animals) during the same period. (*B* and *C*) Comparison of serum LH (*B*) and serum FSH (*C*) levels during aging in wild-type and PACAP$^{-/-}$ males. Levels of LH and FSH hormones were assayed as described in *Materials and Methods*. For LH, 15 wild-type and 10 PACAP$^{-/-}$ males at 4 months of age and 6 wild-type and 9 PACAP$^{-/-}$ males at 15 months of age were used. For FSH, 16 wild-type and 10 PACAP$^{-/-}$ males at 4 months of age and 7 wild-type and 12 PACAP$^{-/-}$ males at 15 months of age were used. (*D* and *E*) Expression profile of StAR and 3β-HSD during aging. Testes of both wild-type ($n = 5$ for 4- and 8-month-old and $n = 3$ for 15-month-old) and PACAP$^{-/-}$ ($n = 6$ for 4-month-old and $n = 5$ for 8- and 15-month-old) animals were dissected and prepared as described in *Materials and Methods*. Level of expression of StAR (*C*) is significantly reduced in wild-type animals when aging but remained low and constant in KO animals. Similar experiments performed with 3β-HSD (*D*) revealed no significant difference in expression levels in wild-type animals during the aging process. In PACAP$^{-/-}$ mice, similar to that of StAR, the level of expression of 3β-HSD was low and constant over time. Data presented in the graph are the results of two independent real-time quantitative PCRs, performed with individual samples run in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Results

PACAP Is Required for Testicular Steroidogenesis *in Vivo*. To test whether the absence of PACAP affects testosterone levels, we measured testosterone in serum collected from 4-month-old wild-type and PACAP$^{-/-}$ male mice (Fig. 1*A*). We recorded a significantly lower level of testosterone in the PACAP$^{-/-}$ animals compared with wild type ($P < 0.001$). This comparatively low testosterone level was consistently correlated with a significantly lower weight of seminal vesicles in PACAP$^{-/-}$ compared with wild-type animals (0.39 ± 0.05 g for wild type, $n = 23$ and 0.325 ± 0.013 g for PACAP$^{-/-}$, $n = 9$; $P < 0.001$).

To explain this dramatic reduction in testosterone levels, we investigated a possible down-regulation of steroidogenesis in testis from KO animals. We compared expression levels of steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase (3βHSD) (two key molecules of steroidogenesis), at 4 and 8 months of age, by real-time quantitative PCR. StAR is a protein which acts outside the mitochondria to induce cholesterol transport across the mitochondrial membranes (17, 25), and 3β-HSD is one of the main steroidogenic enzymes. StAR-expression analysis (Fig. 1*D*) revealed that, at 4 months of age, RNA expression is significantly lower in PACAP$^{-/-}$ mice compared with wild type ($P < 0.001$). The expression level of 3β-HSD RNA (Fig. 1*E*) is also significantly affected in 4-month-old PACAP$^{-/-}$ mice when compared with wild type ($P < 0.01$). In addition to these findings, we showed that the protein level of StAR was lower in young PACAP$^{-/-}$ animals compared with wild type (Fig. 4). Collectively, these results suggest that steroidogenesis is down-regulated in the Leydig cells of young adult PACAP$^{-/-}$ animals, which could account for the observed decrease in serum testosterone levels.

To investigate the origin of the dysfunction (central, at the pituitary level or peripheral, at the testis level), we measured LH (Fig. 1*B*) and follicle-stimulating hormone (FSH) (Fig. 1*C*) serum concentration in wild-type and PACAP$^{-/-}$ male mice at 4 and 15 months of age. No significant difference was detected for either hormone between wild-type and PACAP$^{-/-}$ animals.

Steroidogenesis Remains Constant and at a Low Level During Aging in PACAP$^{-/-}$ Animals Compared with Wild Type. Because testosterone is known to decrease with age (26), we analyzed the serum testosterone level in aged wild-type and PACAP$^{-/-}$ mice (15 months). In wild-type animals, a dramatic decrease was observed during aging ($P = 0.0033$), whereas, in PACAP$^{-/-}$ animals, levels are constant over time at a low level comparable with that seen in older wild-type mice (Fig. 1*A*). We further investigated steroidogenesis in the older animals by measuring the relative expression levels of *StAR* and *3β-HSD* genes by using quantitative real-time PCR. In wild-type animals, StAR expression (Fig. 1*D*) was gradually and significantly reduced during aging ($P = 0.049$ between 4 and 8 months of age, and $P = 0.006$ between 8 and 15 months of age) but not *3β-HSD* expression (Fig. 1*E*). In PACAP$^{-/-}$ mice, expression levels of StAR and *3β-HSD* mRNA were low and constant over time (Fig. 1 *D* and *E*).

Absence of PACAP Delays Testicular Aging. To test whether down-regulation of steroidogenesis could result in protection against testicular aging in the PACAP-deficient mice, we studied the testicular structure at both 4 and 15 months of age to investigate whether low activity in testosterone production in PACAP$^{-/-}$ mice might affect their testicular anatomy.

Histopathological studies of 4-month-old wild-type and PACAP$^{-/-}$ testes revealed no difference in testicular morphology (Fig. 2*A*). Consistent with this finding, there was no difference in the percentage of degenerated seminiferous tubules (Fig. 2*B*) or testis plus epididymis weight (Fig. 2*C*) between PACAP$^{-/-}$ and wild-type animals. Moreover, morphometric parameters in the young PACAP$^{-/-}$ mice are indistinguishable from those of wild-type mice, suggesting that testicular structure is not affected in PACAP$^{-/-}$ animals (Table 1).

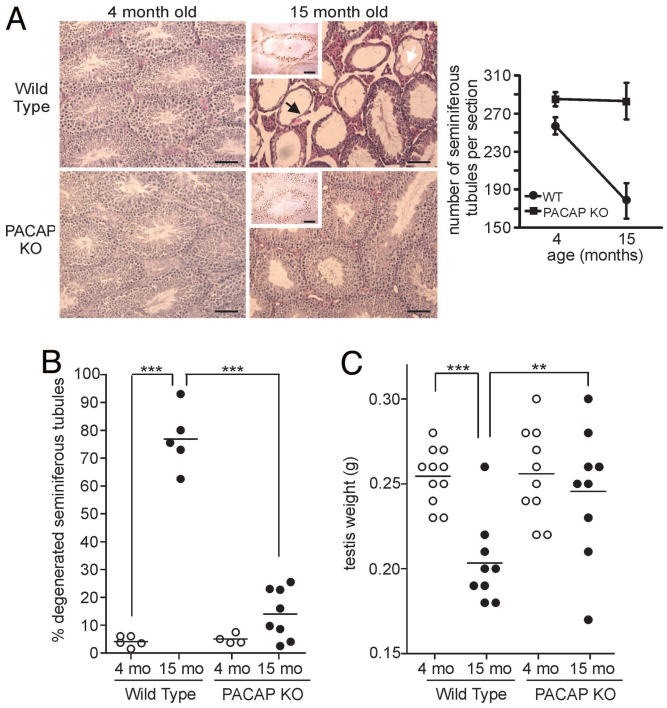

Fig. 2. Histopathology of 4- and 15-month-old wild-type and PACAP-deficient mouse testes. (*A*) Testes of both wild-type mice at 4 ($n = 5$) and 15 ($n = 5$) months of age and PACAP-deficient mice at 4 ($n = 4$) and 15 ($n = 8$) months of age were fixed in 4% paraformaldehyde and embedded into paraffin. Mounted sections (6 μm) were deparaffinized, rehydrated, and stained with hematoxylin and eosin. (Scale bars, 100 μm.) The black arrow shows the depletion of cells often observed in the seminiferous tubules of old wild-type animals, and the white arrow points to the vacuolizations also found at high frequency around the seminiferous tubules of these animals. Note the good preservation of germ cells in the old PACAP$^{-/-}$ testis compared with the wild type. (*Inset*) c-*kit* Staining, confirming the loss of germ cells in the old wild-type testis when compared with the old PACAP$^{-/-}$ testis. (Scale bars, 50 μm.) The associated graph represents the number of seminiferous tubules counted per testis section for the wild-type and PACAP$^{-/-}$ animals. (*B*) Rate of seminiferous tubule degeneration. On three testis sections stained with hematoxylin and eosin (n between 4 and 8 for each group), two populations of seminiferous tubules were scored as explained in *Materials and Methods*. Results are presented as the percentage of degenerated seminiferous tubules per testicular section. (*C*) Testis weight. Testes and epididymis were dissected out and weighed in both wild-type and PACAP-deficient mice at 4 months of age ($n = 11$ and $n = 10$, respectively) and also at 15 months of age ($n = 9$ and $n = 7$, respectively). **, $P < 0.05$; ***, $P < 0.005$.

At 15 months, the structure of wild-type testis (Fig. 2*A*) displayed typical signs of aging: a severe depletion of germ cells and presence of vacuolizations all around the thin epithelium, constituted predominantly of residual Sertoli cells. Akin to changes in the degenerated testes of aging Brown Norway rats, these changes were localized, with some preservation of residual normal seminiferous tubules yet a high percentage of degenerated tubules (77%) (Fig. 2*B*), a significant increase in degeneration when compared with the 4-month-old adult mice. These features were associated with a significant loss in testicular plus epididymis weight (Fig. 2*C*).

In PACAP$^{-/-}$ animals at the same age, the whole architecture of the testis was well preserved (Fig. 2*A*), comparable with that observed in the young PACAP$^{-/-}$ testis. We recorded only a low percentage of degenerating seminiferous tubules, with no significant difference from young adult KO mice (14% in 15-month-old and 6.8% in 4-month-old animals) (Fig. 2*B*). This preservation of the structural integrity of seminiferous tubules is associated with no significant loss of weight in testis plus epididymis, which remains constant from 4 to 15 months (Fig. 2*C*). Results of morphometric analyses showed a strong and significant decrease in the volume of seminiferous tubules and seminiferous epithelium in wild-type testis during aging. This phenomenon was not observed in the aging PACAP$^{-/-}$ testis, because the parameters, volume of seminiferous tubules and seminiferous epithelium, were constant when compared with the young PACAP$^{-/-}$ testis (Table 1).

Peroxynitrite Formation and Apoptotic Testicular Cells Are Decreased in Aged PACAP$^{-/-}$ Animals Compared with Wild Type. To investigate whether ROS formation was reduced in old PACAP$^{-/-}$ mice, we assessed peroxynitrite synthesis in 4% paraformaldehyde-fixed testes by using an antibody against nitrotyrosine, a molecule formed in reaction with nitrite oxide after an oxidative stress. This antibody is commonly used to determine peroxynitrite formation (27), a marker of ROS. In wild-type animals, there was a strong labeling of peroxynitrites inside the seminiferous tubules, particularly in germ cells undergoing later stages of spermatogenesis (Fig. 3*A*), whereas labeling is considerably decreased in the old PACAP$^{-/-}$ testis (Fig. 3*B*).

Because loss of germ cells is mainly due to apoptosis during normal testicular aging (7), and ROS are believed to enhance this process, we examined whether the preservation of spermatogenesis observed in the PACAP$^{-/-}$ mice is the result of inhibition of testicular germ cell apoptosis. To do so, we assessed *in situ* the presence of apoptotic cells in 15-month-old PACAP$^{-/-}$ and wild-type mice by using the TUNEL method (Fig. 3 *C* and *D*).

In wild-type testes (Fig. 3*C*), we focused on the small areas containing "healthy" tubules (among the overall 23% of nondegenerated tubules) to assess whether germ cell apoptosis was occurring within anatomical structures still containing living cells. As expected, we observed a high frequency of TUNEL-positive apoptotic germ cells. At the same age, in PACAP$^{-/-}$ mice, only a few TUNEL-positive cells were detected (Fig. 3*D*).

In PACAP$^{-/-}$ Mice, StAR and P450c17 Protein Levels Are Partially Rescued After PACAP Injection. To rescue the defect in testosterone biosynthesis seen in the young PACAP$^{-/-}$ animals, a group of PACAP$^{-/-}$ mice (4 or 15 months old) received daily i.p. injections of 2 nmol of PACAP for 10 days. Although serum testosterone measurements failed to show a significant increase in the KO animals (data not shown), the active form of StAR protein (37 kDa)

Table 1. Morphometric analysis in young and old PACAP$^{-/-}$ testis compared with wild-type

Animals	Volume of seminiferous tubules (μl per testis)	Volume of interstitium (μl per testis)	Volume of seminiferous tubule lumen (μl per testis)	Volume of seminiferous epithelium (μl per testis)	Volume of Leydig cells (μl per testis)
Young wild type (4 mo)	99.52 ± 3.58	7.97 ± 0.63	11.53 ± 0.65	88 ± 3.24	3.97 ± 0.19
Young PACAP$^{-/-}$ (4 mo)	96.72 ± 3.16	5.77 ± 0.73	10.18 ± 0.79	86.53 ± 2.76	3.81 ± 0.53
Old wild type (15 mo)	50.705 ± 0.42	9.29 ± 0.42	16.91 ± 2.79	33.8 ± 2.36	6.3 ± 0.05
Old PACAP$^{-/-}$ (15 mo)	107.63 ± 14.49*	8.5 ± 1.67	16.96 ± 2.1	90.67 ± 12.54*	5.24 ± 1.13

From 4% paraformaldehyde-fixed tissue, morphometric analysis was performed as described in *Materials and Methods*. A number of young ($n = 5$) and old ($n = 2$) wild-type animals and four young and four old PACAP$^{-/-}$ animals were analyzed. *, $P < 0.05$; significant difference between old wild-type and old PACAP$^{-/-}$ mice.

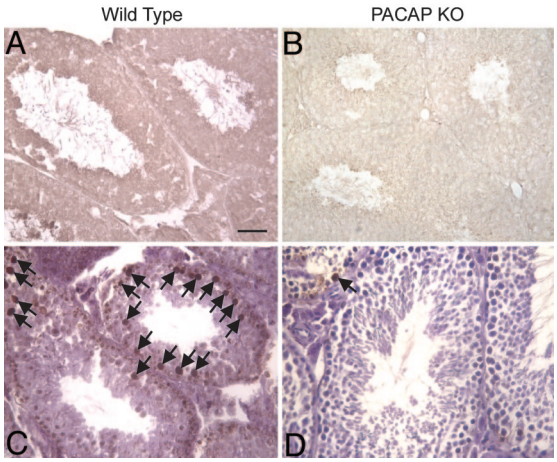

Fig. 3. Peroxynitrite formation and testicular apoptosis in 15-month-old wild-type and PACAP$^{-/-}$ mice. (*A* and *B*) Peroxynitrite formation. Two testis sections of 15-month-old wild-type ($n = 3$) and 15-month-old PACAP$^{-/-}$ ($n = 3$) mice were stained with the anti-nitrotyrosine antibody to reveal the degree of peroxynitrite formation. Three different controls were included for each experiment: without the primary antibody, without the secondary antibody, and without primary and secondary antibodies, all showing a complete absence of staining (data not shown). Note the strong staining of peroxynitrites found in the old wild-type testis, in contrast to the moderate staining in the PACAP$^{-/-}$ testis. (Scale bar, 50 μm.) (*C* and *D*) Representative examples of apoptotic germ cells detected by the TUNEL method. *In situ* detection of cells with DNA strand breaks was assessed in three testis sections from 15-month-old wild-type (*Left*; $n = 4$) and PACAP$^{-/-}$ (*Right*; $n = 5$) mice, as described in *Materials and Methods*. For negative controls (data not shown), terminal deoxynucleotidyl transferase enzyme was substituted with PBS. Sections were counterstained with methyl green. Black arrows show the localization of apoptotic cells in the seminiferous tubules. (Scale bar, 50 μm.)

increased 4.5-fold after PACAP injections in the 4-month-old animals compared with vehicle (water), resulting in a partial rescue of 38% compared with the wild-type value. For P450c17, there was an increase of 1.9-fold after PACAP injection, resulting in a partial rescue of 31% of the wild-type phenotype (Fig. 4). In old animals, levels of P450c17 protein were increased, but no difference was observed for StAR, between the treated and nontreated animals.

Discussion

Age-related decline in male sex hormones affects a large proportion of the aging population (1). There is an intense controversy about the benefit/risk ratio of testosterone replacement therapy in aging men (3). One of the multiple reasons for this lack of general agreement resides in the poor understanding of the basic biological mechanisms of testicular aging. It has been suggested that steroidogenesis itself and, more specifically, the microsomal P450c17 protein (5, 6) participates in the age-induced testis degradation by generating ROS, which have been shown to negatively impact on steroidogenesis *in vitro* (28) and have been proposed as responsible for the age-related senescence of the Leydig cells *in vivo* (14).

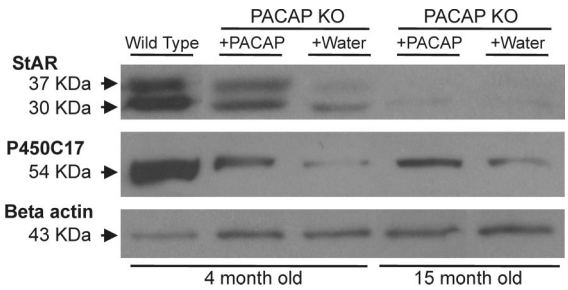

Fig. 4. Comparison of StAR and P450c17 protein levels by Western blots after rescue experiment. A rescue experiment was performed as described in *Materials and Methods*. StAR and P450c17 protein levels were assessed in two 4-month-old and two 15-month-old PACAP$^{-/-}$ animals that received i.p. injections of 2 nmol of PACAP (+PACAP) and compared with the levels of the same proteins in two 4-month-old and 15-month-old testes from PACAP$^{-/-}$ animals that received i.p. injections of water (+Water) as a control. Shown is the representative intensity of the blots seen for each group of animals.

The neuropeptide PACAP has been implicated in regulation of testosterone level in cultured Leydig cells (20–22, 29). It has also been shown to potentially enhance the release of LH *in vivo* (18). PACAP, acting on PAC1 receptors that efficiently enhance cAMP production and intracellular calcium mobilization was shown to directly interact with many cell types, such as gonadotrope, Leydig, germ, and Sertoli cells (16).

In this study, we first showed that, at 4 months of age, PACAP$^{-/-}$ mice display lower serum testosterone concentration and lower levels of steroidogenic enzymes compared with age-matched controls. However, these constant low levels of testosterone did not impair spermatogenesis, consistent with similar findings in the LH receptor KO mice, where intratesticular testosterone is suppressed to 2% of wild type, yet LH-independent autonomous steroidogenesis is sufficient to maintain spermatogenesis (30).

Our findings that PACAP is required for testosterone biosynthesis *in vivo* are consistent with previous *in vitro* studies in which PACAP was shown to stimulate testosterone secretion by embryonic and adult Leydig cells (20–22, 29). Nevertheless, the low testosterone concentration in PACAP$^{-/-}$ mice does not affect the fertility of these animals that routinely breed in our facility. Moreover, we showed that the normal testicular architecture of these animals at 4 months of age is indistinguishable from their wild-type littermates, suggesting that some intratesticular testosterone is still present to ensure a normal spermatogenesis. It is known that testosterone is essential for normal spermatogenesis, but questions remain about the minimum levels required, which varies by species. In mice, only minimal amounts of testosterone are required to maintain spermatogenesis (30), whereas suppression of steroidogenesis in rat, monkey, and human is the basis for the first generation of hormonal contraceptives (31). In addition, studies performed with female PACAP$^{-/-}$ mice showed that fertility is affected in these animals (32), raising the possibility that PACAP is likely to be an important regulator of reproductive functions in both males and females.

Our results do not show any difference in LH and FSH concentration in young PACAP$^{-/-}$ mice. A number of studies suggest that PACAP could exert a direct action on Leydig cells (20–22, 29), supporting the argument for a peripheral dysfunction at the testis level. Therefore, we expected higher levels of LH in PACAP$^{-/-}$ mice compared with wild-type animals. There are two main explanations for the lack of high levels of LH expected in primary hypogonadism: (*i*) residual testosterone, although low, could still be high enough to ensure negative pituitary feedback, and (*ii*) the normal levels of LH, given the low testosterone values, are actually inappropriately low, giving credence to the concept that PACAP may stimulate LH release *in vivo* as suggested in ref. 18. In this context, the absence of PACAP prevents the appropriate and expected surge in LH levels in the context of testosterone deficiency in PACAP$^{-/-}$ mice. This latter explanation supports the argument that the observed low testosterone levels in PACAP$^{-/-}$ animals could be caused by a mixed (central and peripheral) defect. In this case, experiments in which KO animals are stimulated by gonadotropin-releasing hormone before measure of LH/FSH production may prove helpful to identify the origin of the dysfunction. These experiments are beyond the scope of this investigation, which focuses on the effect of PACAP on aging.

To further elucidate the origin of defective steroidogenesis in young mutant males, we focused on Leydig cell function. By analyzing levels of expression of StAR and 3β-HSD RNA, we

demonstrated a down-regulation of these two key components of testosterone biosynthesis in 4-month-old PACAP$^{-/-}$ mice compared with wild type. Moreover, we showed that StAR and P450c17 protein levels were lower in the 4-month-old PACAP$^{-/-}$ testis. These *in vivo* data are consistent with previous findings in cultured Leydig cells showing that PACAP stimulates testosterone biosynthesis and cAMP production (20, 22). In turn, cAMP regulates the expression of steroidogenic enzymes (17). Therefore, we speculate that the lack of PACAP in germ cells precludes activation of PAC1 receptors normally expressed in Leydig cells and the subsequent cAMP accumulation, which normally enhances expression of several genes responsible for testosterone biosynthesis.

The decline we observe in testosterone levels, StAR expression, and integrity of testicular architecture in aging wild-type mice is consistent with previous aging studies performed in rodents. In the Brown Norway rat, testosterone levels and StAR expression were also reduced with age (26, 33), whereas 3β-HSD remained constant (34). In aging CBA/Ca mice (20 months of age), alterations in testis size and seminiferous tubule structure as well as germ cell depletion were also observed (35). In aged PACAP$^{-/-}$ mice, we observed a remarkable absence of testicular aging. However, the apparent maintenance of testicular structural integrity does not address the question of the fertility of PACAP$^{-/-}$ mice at the age of 15 months.

Here, we have shown that, at 15 months of age, apoptotic cells are more abundant in wild-type than in PACAP$^{-/-}$ mice, establishing a likely mechanism for the cell loss associated with testicular aging. Moreover, peroxynitrite formation was decreased in these old PACAP$^{-/-}$ testes compared with age-matched wild-type testes, suggesting that slowing of testicular aging occurs through a mechanism that decreases ROS formation.

Steroidogenesis itself and, particularly, P450c17 protein has been shown to be a potential source of ROS generation in the testis (35). Because PACAP$^{-/-}$ mice have decreased P450c17 activity, we propose that testicular aging is slowed in PACAP$^{-/-}$ mice because of reduced steroidogenesis and the consequent reduction in ROS formation. Consistent with this hypothesis, transgenic mice exhibiting a dominant-negative mutation in ubiquitin show a testicular phenotype similar to our PACAP$^{-/-}$ mice due to a lack of protein degradation by the 26S-proteasome (36). In that model, the authors suggest that the level of steroidogenesis and, therefore, ROS formation is lower in these mutant mice compared with wild type, because damaged steroidogenic enzymes are not degraded.

Alternatively, PACAP, a peptide known to control the proliferation and differentiation of several cell types (16), could directly influence the lifespan of germ cells. In addition, it has been shown that PACAP$^{-/-}$ mice present numerous phenotypes, including high mortality of PACAP$^{-/-}$ mice (37), inadequate heat production (38), reduction in glucose levels (39), and deficits in circadian light response (40). It is possible that these factors could influence testicular aging as well. In particular, caloric restriction is well known to reduce circulating glucose, which could inhibit reproductive capability (41).

We propose a model in which the absence of PACAP results in protection against testicular aging (Fig. 5). In wild-type animals, PACAP stimulates steroidogenesis in Leydig cells by stimulating LH release at the pituitary level but also directly, at the testis level. Consequently, ROS are generated as a byproduct, which leads to testicular germ cell death by apoptosis and ultimately causes testicular aging. The absence of PACAP results in down-regulation of testosterone biosynthesis, which leads to diminished ROS generation and consequent protection against apoptosis-mediated testicular aging.

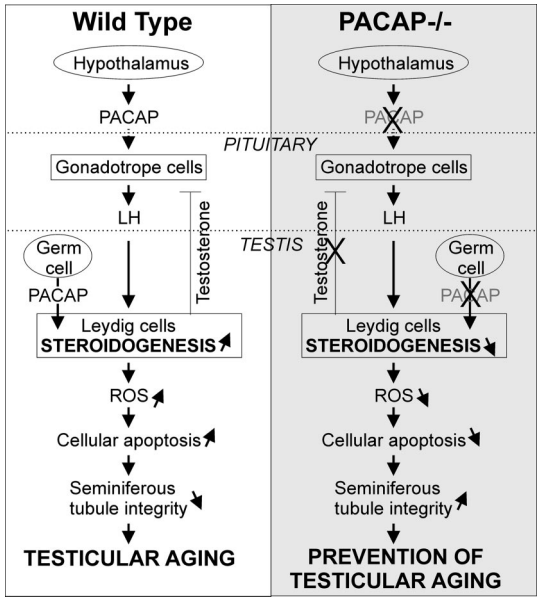

Fig. 5. Schematic representation of putative mechanisms leading to protection against testicular aging in PACAP$^{-/-}$ mice. In wild-type animals (*Left*), PACAP produced by the hypothalamus stimulates the release of LH at the pituitary level. In testis, PACAP is also synthesized by germ cells. Both PACAP and LH act on Leydig cells, to enhance testosterone biosynthesis. Generation of ROS directly results from steroidogenesis. These free radicals, by inducing apoptosis in testicular cells, alter the architectural integrity of seminiferous tubules and ultimately cause testicular aging. Conversely, in PACAP$^{-/-}$ animals (*Right*), steroidogenesis is down-regulated in Leydig cells. Consequently, fewer ROS are produced, and germ cell apoptosis is inhibited. Finally, this mechanism leads to a protection against testicular aging in PACAP$^{-/-}$ mice.

Materials and Methods

Animals. PACAP-deficient male mice generated and backcrossed for six generations in defined C57BL/6 genetic background (40) were used in the study. For controls, wild-type mice of matching genetic background and age were analyzed.

Blood Collection and Hormone Measurements. Mice were anesthetized with isoflurane and immediately dissected to access the heart. Blood was collected and spun down at 10,000 $\times$ *g* for 10 min at 4°C to isolate the serum. If the serum was not used immediately, it was kept at −20°C until further analysis.

Serum testosterone concentrations from individual mice were measured by radioimmunoassay after ether extraction according to published methodology (42). The sensitivity of the assay was 7.8 pg per tube, the inter-assay variation was 8%, and the intra-assay variation was 4.3%.

Serum LH and FSH levels were measured by the National Hormone and Peptide Program (A. F. Parlow, University of California, Los Angeles) using mouse LH and FSH radioimmunoassay kits. The sensitivity for the LH assay was 0.3 ng/ml. The sensitivity for the FSH assay was 2.0 ng/ml (www.healthsystem.virginia.edu/internet/crr/methodspage.cfm).

Quantitative RT-PCR. Testes were removed, weighed, quick frozen in liquid nitrogen, and homogenized in TriZol solution. Total RNA from tissues was isolated by using the chloroform extraction method and subjected to DNase I treatment (30 min at 37°C) to remove genomic DNA contamination. Samples of total RNA (1 μg per sample) were reverse-transcribed into cDNA according to manufacturer's procedures (Iscript kit; Bio-Rad). Real-time quantitative PCR was performed by using Sybr green PCR supermix from Bio-Rad in combination with primer sets designed as described in ref. 43. PCR amplifications were carried out at 45 cycles (96°C for 20 s, 62°C for 20 s, and 72°C for 20 s) before the melting-curve assay. By using the following primer sets for amplification of StAR gene (5′-TTCTCAACTGGAAGCAACACT-3′ and 5′-CTTCTGCATAGCCACCTCTC-3′) and 3β-HSD (5′-GGATCATCAAGATGTTGGTGC-3′ and 5′-TGGTTTCTGGTCGGAATACTT-3′), we were able to selectively amplify DNA fragments of 75 and 90 bp

of the National Center for Biotechnology Information-published StAR and 3β-HSD mouse sequences (accession nos. NM_011485 and NM_008293). Standardization of basal levels between samples was made possible by using GAPDH as housekeeping gene (43). Specificity of gene amplification was attested to by both melting curves and sequencing of PCR products.

Histopathology and Morphometric Analysis. To determine the testicular structure of PACAP$^{-/-}$ mice, testes from wild-type and PACAP$^{-/-}$ mice were weighed and fixed in 4% paraformaldehyde. Tissues were then embedded in paraffin before sectioning. Mounted sections (6 μm) were stained with hematoxylin and eosin according to standard protocols. In three sections per animal, seminiferous tubules were counted as "healthy" if the general architecture showed normal spermatogenesis with all the layers of spermatogenic cells associated with each of the 12 stages of spermatogenesis. Conversely, they were counted as "degenerated" if they showed depletion of all or some germ cell layers usually associated with vacuolizations within the seminiferous tubule. This quantification was performed in a blind study by two independent investigators.

The volume densities (Vv) of seminiferous tubules, tubular lumens, interstitium, and Leydig cells were determined by the point-counting method (44, 45). Five randomly selected sections per animal in each group were examined by using an American Optical microscope with a $\times$40 objective and a $\times$10 eyepiece fitted with a square lattice containing 121 intersections. The results were expressed as a percentage of the testis volume. The absolute volume of each of the testis components was then obtained by multiplying its Vv by fresh testis volume (Vv%).

Immunohistochemistry. Sections were deparaffinized and rehydrated in serial alcohol baths. For nitrotyrosine staining, but not for c-*kit*, sections were first subjected to antigen retrieval in citrate buffer (pH 6.0, 10 min at 95°C) and then quenched for endogenous peroxidase activity with 2% H_2O_2 for 10 min. Sections were incubated overnight at 4°C in a humidity chamber with a nitrotyrosine antibody (10 ng/ml; Upstate Biotechnology, Lake Placid, NY) to evaluate peroxynitrite formation or a c-*kit* antibody (1:2,000; Santa Cruz Biotechnology) to mark germ and Leydig cells. The detection was made possible by using the ABC complex (Calbiochem) and 3,3 diaminobenzidine.

Western Blot. Testes were solubilized in a 1% KCl/1 mM EDTA buffer supplemented with a mixture of protease inhibitors. Proteins were separated by electrophoresis and transferred to a poly(vinylidene difluoride) membrane. After incubation in 10% blotting grade blocker (Bio-Rad) in PBS-T for 2 h, membranes were incubated overnight at 4°C with either a rabbit anti-porcine P450c17α antiserum (1:7,000) or a rabbit anti-mouse StAR antiserum (1:7,000). These two antisera were generously provided by Dale Buchanan Hales (University of Illinois, Chicago). The mouse anti-actin antibody (1:10,000; Sigma) was used as the loading control. Membranes were then incubated with the secondary antibody [goat anti-rabbit horseradish peroxidase (HRP), 1:10,000, or anti-mouse HRP, 1:6,000], for 1 h. Signal was detected by using a chemiluminescence kit (ECL; Amersham Pharmacia Biotech).

Apoptosis. From 4% paraformaldehyde-fixed sections, apoptotic cells were detected *in situ* by using the TUNEL technique (Apop Tag-peroxidase kit; Intergen) according to the manufacturer's instructions.

Rescue Experiment. A rescue experiment was performed at 4 and 15 months of age. Two groups of mice were studied: control mice, which received water injections; and treated mice, which received daily i.p. injections of 2 nmol of PACAP38 (Calbiochem). After treatment, the mice were killed, and the testes were dissected out and quick frozen in liquid nitrogen to extract proteins and measure the level of P450c17 and StAR.

Data Evaluation and Statistical Analysis. Results were expressed as the average $\pm$ SEM. Two-way analyses of variance, with genotype (wild type versus PACAP KO) and age (4, 8, or 15 months of age) as the between-subject factors, were used with nearest neighbor variables by using the program GRAPHPRISM4. When the overall analysis was significant, multiple pairwise post hoc analyses using Student *t* tests and Bonferroni/Dunn adjustments were conducted to determine where the significant differences lay. The family-wise critical significance level was set at 0.05.

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