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Intrapituitary Adenoviral Administration of 7B2 Can Extend Life Span and Reverse Endocrinological Deficiencies in 7B2 Null Mice

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The prohormone convertase PC2 requires the aid of a helper protein, known as 7B2, for production of active enzyme. Deletion of 7B2 results in a lethal phenotype resembling Cushing's disease. In this study, we have investigated the effect of a single low dose of recombinant adenovirus vector encoding 7B2 and delivered directly to the pituitary of 7B2 nulls on pituitary ACTH, plasma ACTH, corticosterone, αMSH and glucose, and survival time. We show that after injection of recombinant adenovirus encoding 27-kDa 7B2 into 7B2 nulls, transgene expression, as measured by RIA for 7B2, exhibits a transient elevation in the pituitary and blood, with a slight but significant elevation of PC2 activity in pituitaries of 7B2 nulls and a drop in the level of circulating ACTH concomitant with a small increase in circulating αMSH. The level of circulating blood glucose was increased, and that of corticosterone was decreased. Lastly, slight but significantly prolonged survival times were observed. These data showing partial rescue of 7B2 nulls support the idea that adenoviral administration of 7B2 will represent an effective means to study the role of this interesting neuroendocrine protein on endocrine function *in vivo*. (*Endocrinology* 143: 2314–2323, 2002)

RECOMBINANT ADENOVIRAL VECTORS represent a highly efficient means to accomplish *in vivo* gene transfer for the investigation of the biological function of gene products (1). Adenoviruses have found their place as a useful tool for gene transfer to the pituitary gland with the aim of providing therapeutic treatment for pituitary disease and another endocrinopathies under pituitary control (1–3).

The prohormone convertases 1 and 2 (PC1 and PC2) belong to the group of subtilisin-related proteolytic enzymes that accomplish neuroendocrine-specific cleavages of larger precursors, forming biologically active peptide hormones and neuropeptides (4). PC1 and PC2 are specific to neuroendocrine cells and account for much of this cleavage activity. The small neuroendocrine protein 7B2, discovered in 1982 (5), interacts with the prohormone convertase PC2 in the regulated secretory pathway (6–9). In neuroendocrine cells the 7B2 protein forms a complex with proPC2 in the endoplasmic reticulum; complex formation then results in both increased transport of proPC2 to the Golgi and facilitation of proPC2 maturation (9). In the absence of 7B2, proPC2 maturation to an active enzyme does not occur, and an inactive form of PC2 is instead generated. The 27-kDa 7B2 protein is itself a precursor protein and is cleaved during transit in the secretory pathway into two fragments: an amino-terminal domain (21-kDa 7B2) and a carboxyl-terminal domain of 31 residues (reviewed in Refs. 4 and 10). Intact 7B2 and its carboxyl-terminal domain represent potent inhibitors of PC2 (11, 12). Thus, 7B2 represents a bifunctional protein that can either facilitate or inhibit PC2 activity; *in vivo*, the facilitatory effect predominates (9).

Using a novel transposon-based technique, Westphal *et al.* (13) have generated 7B2 null mice. The null mutation in the 7B2 locus was found to result in a lethal phenotype and severe endocrine abnormalities that in many, though not all, respects resemble pituitary Cushing's. 7B2 null mice lack PC2 activity; multiple metabolic alterations, similar but not identical to those found in PC2 null mice, have been demonstrated (13). Unexpectedly, and quite differently from PC2 null mice, 7B2 null mice exhibit extremely high levels of circulating ACTH of intermediate lobe origin, indicating a possible novel role for 7B2 in the normal control of secretion of peptides from this lobe (13). 7B2 null mice possess constant hypoglycemia as well as a high level of circulating corticosterone and proinsulin, little or no circulating glucagon, adrenocortical hypertrophy (13), and anterior pituitary hypotrophy with a lack of POMC expression in the anterior lobe (13a). Given that the only known role of 7B2 to date is as a binding partner for PC2, the question of why 7B2 null mice but not PC2 null mice exhibit a lethal disease state is intriguing. The disease is not due to alterations during development because adrenalectomy of 3-wk-old mice is able to rescue animals from the lethal phenotype (13a). Generally, Cushing's disease in childhood is a rare pathology and has certain different clinical and therapeutic characteristics than Cushing's in adults.

In this report we have compared the responses of 7B2 null and WT mice after delivery of a single low dose of a recombinant adenoviral vector encoding 27-kDa 7B2 [or β galactosidase (β gal) as a control adenoviral vector]. Our results

Abbreviations: AMC, Aminomethylcoumarin; β gal, β galactosidase; HPA, hypothalamus-pituitary-adrenal; PC2, prohormone convertase 2; pfu, plaque-forming units; RAd, recombinant adenoviral vector; 7B2 null mice, mice with a null mutation in the 7B2 gene; WT, wild-type.

demonstrate that injection of a low number of infectious units of an adenovirus encoding 27-kDa 7B2 produces a transient beneficial response in the levels of circulating corticosterone, αMSH, blood glucose, and pituitary ACTH as well as a small increase in pituitary PC2 activity.

Materials and Methods

Production of recombinant adenoviruses (RAds)

The shuttle vector plasmids contain expression cassettes containing the cytomegalovirus immediate early promoter controlling the expression of the 7B2/21-kDa and 7B2/27-kDa sequences and an SV40 polyadenylation sequence. The expression cassettes are surrounded by Ad5 sequences from 1 to 455 bp on the left side and 3334 to 6103 bp on the right side. RAds were generated by cotransfection with each shuttle vector and pBHG10 (Microbix Biosystems, Toronto, Canada), which comprises a circular unpackageable form of the adenovirus type 5 genome, with deletions in the E1 and E3 regions into human embryonic kidney 293 cells, using the calcium phosphate precipitation method (14). Production of high-titer stocks, purification with the fluorocarbon compound Arklone P (Basic Chemical Co. Ltd., High Wycombe, Bucks, UK), plaque purification and titration of RAds during construction were carried out as described by Thomas *et al.* (15).

Viral DNA was obtained as described by Revah (16). To confirm the presence of the transgenes, viral DNA digestion with *Hin*dIII and subsequent Southern blot hybridization was performed using specific probes against the transgenes, labeled by random priming with digoxigenin-dUTP (Roche Molecular Biochemicals, East Sussex, UK).

Viral stocks were assayed for the presence of replication-competent adenovirus using a replication competency assay by the supernatant rescue assay (17) and plaque assayed. Viruses were also assayed for the presence of endotoxin (lipopolysaccharide) using the E-TOXATE assay (Sigma-Aldrich Corp., St. Louis, MO) according to the manufacturer's protocol. The viruses used were designated endotoxin free as defined by Cotten *et al.* (18). The concentrations of endotoxin in viral stocks were $3–6 \times 10^{-4}$ endotoxin units per dose (where 0.06 EU/ml represents a positive result) of adenovirus injected (5×10^6 plaque-forming units [pfu]/5 μl).

Isolation of RAds for use in this study

The 911 cell line was used for routine purification of adenoviruses. Cells were grown in DMEM/F12 medium containing 5% FCS and 1% penicillin and streptomycin in 20 150-mm dishes until confluence reached 95%. Cells were washed with prewarmed 1× PBS, infected with adenovirus, and incubated until just before detachment from the dish in a 37 C, 6% CO_2 incubator. Cells were pelleted by centrifugation at 4 C and the supernatant removed. The pellet was frozen and thawed three times using a dry ice/ethanol bath and a 37 C water bath. Double cesium chloride gradient centrifugation was performed on cell extracts using SW41 rotor (Beckman Instruments, Inc., Palo Alto, CA) for 2 h (for the first centrifugation) and for 18 h (for the second centrifugation) at 30,000 rpm at 4 C. After isolation of the viral band, cesium chloride was removed using Sepharose CL-4B spin columns. The adenoviral particles were stored in 5% sucrose in virus storage buffer (150 mM NaCl; 20 mM HEPES, pH 7.8) at −70 C.

Antiadenovirus-neutralizing antibody assay

Serum or plasma was prepared from blood taken every second day until 14 d post viral injection. The presence of circulating antiadenovirus neutralizing antibodies was assayed by heat-inactivating sera at 56 C for 30 min before 2-fold serial dilution in MEM medium containing 2% FCS. Each dilution of serum was then incubated in duplicate with 10^6 infectious units of RAd β gal in 10 μl for 90 min. Fifty microliters of 2×10^4 human embryonic kidney 293 cells were placed per well plated in a 96-well plate at 37 C for 1 h. Fifty microliters of MEM with 10% FCS were then added to each well. Cells were left at 37 C for 20 h. Following this incubation period, cells were stained using a β gal staining kit (PanVera, Madison, WI). Titers were taken as the reciprocal of the serum dilution factor that caused approximately 50% inhibition of histochemical staining of β gal staining, compared with controls.

Animals

Three-week-old mice of the strains FVB (a 7B2 wild-type, WT) and 129/Sv (7B2 WT and 7B2 null) derived from crosses of $7B2^{het/het}$ parents (13) were used. All animals had free access to food and water, a photoperiod of 12 h light alternating with 12 h dark, and housing under constant temperature and humidity. Animals were anesthetized ip with ketamine/xylazine (10 μl/10 g of body weight) to produce deep anesthesia for intrapituitary viral delivery or Avertin (2.5% vol/vol in 0.9% NaCl, 0.015–0.020 ml/g body weight) was used for venopuncture from the retroorbital plexus and cardiac venopuncture performed following intrapituitary injection of recombinant adenoviral vectors. Venopuncture was performed every second day in the time course and pfu response experiments, and every 3–5 d for experiments with 7B2 null and WT animals. Animals were monitored for general health and mortality for 24 h during the postdelivery period and in the recovery process. Animal care and use procedures were approved by the Louisiana State University Health Sciences Center Animal Care Unit.

As a control for virus localization, we used the dye Lissamine Green B (Sigma, St. Louis, MO) for test injections. On necropsy of control experimental animals injected with dye, a green color was detected in and around the pituitary; in the meninges; and at the base of brain, in the hypothalamic region. To examine potential expression of virus in the hypothalamus, we also followed the expression of β gal in the hypothalamus of WT mice injected with control RAd encoding β gal 2 wk after injection. The background signal of β gal in the hypothalamus of untreated mice was 1.3 ± 0.1 mU/hypothalamus (n = 4), whereas in mice treated with control RAd β gal, we detected 2.9 ± 0.8 mU/hypothalamus (n = 4; $P < 0.05$). These quantities represent approximately three times less than the amount detected in pituitaries from the same animals (0.9 ± 0.1 mU/pituitary, n = 4, in the untreated group and 7.8 ± 0.4 mU/pituitary, n = 4, in mice treated with control RAd), indicating reasonably precise delivery of adenovirus.

In vivo delivery of recombinant adenoviral vectors to the pituitary

The procedure for *in vivo* delivery recombinant adenoviral vectors to the pituitary glands was adapted from a hypophysectomy technique for rats and mice by Riley *et al.* (19). This technique involves direct transauricular injection with a 1-ml syringe and 30-gauge needle under deep anesthesia by ketamine/xylazine. Injection volumes of 5 μl or less of 10^6 infectious units for all types of viruses (RAd7B2/27 kDa, RAd7B2/21 kDa, and RAd-β gal and RAd-empty) were required to avoid acute and lethal increases in intracranial pressure. After viral delivery all animals were under intensive observation; recovery from anesthesia proceeded smoothly. No obvious adverse side effects of viral injection were observed 24 h after injection.

PC2 enzyme assay

Frozen pituitaries were homogenized by direct sonication in 150 μl of 0.1 M sodium acetate buffer, pH 5.0, 1% Triton X-100, 1 μM pepstatin, 1 μM E-64, and 1 mM phenylmethanesulfonyl fluoride. Samples were centrifuged at 10,000 rpm for 2 min at 4 C. PC2 enzyme assay was performed in a microtiter plate. The assay buffer consisted of 0.1 M sodium acetate buffer, pH 5.0, 10 mM $CaCl_2$, one-tenth volume of 10× inhibitor mix (56 mM tosylysyl chloromethyl ketone, 60 mM tosylphenylalanine chloromethyl ketone, 20 μM *trans*-epoxysuccinic acid, and 20 μM pepstatin), and 0.5% Triton X-100. The final volume of the reaction after the addition of substrate ([pGlu-Arg-Thr-Lys-Arg-methylcoumarin amide (Peptides International, Louisville, KY) at a final concentration of 0.2 mM] was 50 μl/well (40 μl mix and 10 μl clarified sample). The kinetics of substrate hydrolysis were measured using a microtiter plate fluorometer (LabSystems, Helsinki, Finland) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The amount of released aminomethylcoumarin (AMC) was calculated by reference to a free AMC standard. Results are the means of values derived from three to six pituitaries per group ± SD; values indicate activity per pituitary during the 2-h incubation period. A one-micromolar concentration of the 7B2 CT peptide (human 7B2 155-185) (a specific PC2 inhibitor) was used in duplicate samples to verify the enzymatic activity

as PC2; only the activity inhibited by CT peptide (80–90% of total activity) is shown.

Serum corticosterone, αMSH, and blood glucose assays

Serum for corticosterone analysis was prepared from blood obtained by venopuncture between 1100 h and 1300 h. Sera were stored at −70 C until the assay procedure. The ImmuneChem [^{125}I] Corticosterone RIA (ICN Pharmaceuticals, Inc., Costa Mesa, CA), specifically designed for use in rodents, was used for corticosterone measurements, in duplicate. For glucose analysis we used a standard glucometer. Blood samples for glucose analysis were collected by venopuncture of the lateral tail vein. Plasma for αMSH analysis was prepared from trunk blood and collected in tubes containing 0.1 M EDTA. The samples were cooled in an ice bath immediately, and plasma was separated by centrifugation at 4 C, collected into tubes (Eppendorf, Brinkmann Instruments Inc., Westbury, NJ) and stored at −70 C until the assay procedure. Plasma samples were analyzed for αMSH content by RIA following the manufacturer's instructions (Euro-Diagnostica kit, IBL, Hamburg, Germany).

Pituitary and blood ACTH assay

Twenty 7B2 null animals and 15 7B2 WT animals received intrapituitary injections of RAd7B2/27 kDa or RAd β gal; 14 d later animals were killed and pituitaries individually homogenized via sonication in 150 μl ice-cold 1 N acetic acid, 20 mM HCl, and 0.1% β-mercaptoethanol. The samples were aliquoted and stored frozen at −70 C before thawing and assaying. Samples were subjected to centrifugation for 10 min at 13,000 rpm in a microfuge at 4 C. The clear supernatants were transferred to new tubes and stored at −75 C. Plasma samples were obtained from collection of trunk blood with one-tenth volume of 0.1 M EDTA, pH 8, added as anticoagulant after rapid decapitation between 1100 h and 1300 h. RIAs were performed in duplicate for ACTH in pituitary extracts and plasma using the human ACTH 1-39 assay kit (Nichols, San Juan Capistrano, CA) according the manufacturer's protocol. The assay is specific for intact ACTH 1-39 and does not recognize the PC2 cleavage products corticotropin-like intermediate lobe peptide + MSH.

Pituitary and plasma 7B2 levels, β gal activity

Pituitary extracts and plasma were prepared as described above for the pituitary/plasma ACTH assay. The RIA for 7B2 was described previously (9). Assay of β gal in pituitaries was accomplished using an assay system (Promega Corp., Madison, WI) according the manufacturer's protocol. Total protein was measured using the BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

Statistical analysis

Data were reported as the mean ± SD, analyzed by means of *t* test. Symbols used are: *, $P < 0.05$; **, $P < 0.001$; and ***, $P < 0.0001$.

Results

Time course of 7B2 transgene expression after intrapituitary delivery

The efficiency of recombinant adenoviral vector-mediated 7B2 gene transfer to the pituitary was assessed after injection with RAd 7B2/27 kDa and RAd 7B2/21 kDa (5×10^6, 10^7, and 10^8 pfu/injection) (Fig. 1, A and B) and as a control RAd β gal (Fig. 2, A and B) in FVB 7B2 WT mice. The designation 21 kDa 7B2 represents the 7B2 protein that lacks the C-terminal inhibitory peptide; we used 7B2/27 kDa in the time course and subsequent experiments because it represents the natural replacement protein. The expression of transgenic 7B2 was detected using a 7B2 RIA (9); this assay measures both forms of 7B2.

Low doses (5×10^6 pfu/injection) of Rad 7B2/27 kDa generated more consistent effects on transgene expression than the higher doses of 10^7 and 10^8 pfu/injection. After

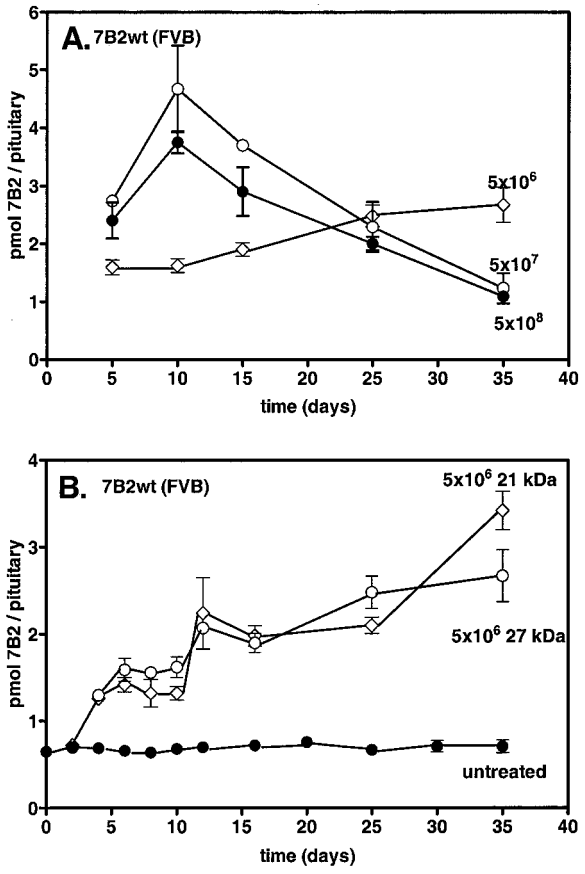

FIG. 1. Time course of 7B2 transgene expression in FVB mice; comparison of 7B2 forms. Time course of 7B2 transgene expression (A): *open circles*, animals injected with RAd 7B2/27 kDa at 5×10^7 pfu/injection; *open diamonds*, animals injected with RAd 7B2/27 kDa at 5×10^6 pfu/injection; and *filled circles*, animals injected with RAd 7B2/27 kDa at 5×10^8 pfu/injection. Comparison of RAd 7B2/27 kDa and RAd 7B2/21 kDa (B): *open circles*, animals injected with RAd7B2/27 kDa at 5×10^6 pfu/injection; *open diamonds*, animals injected with 7B2/21 kDa at 5×10^6 pfu/injection; and *filled circles*, untreated controls. 7B2 levels were measured by RIA. Each point represents the mean ± SD of four to five mice per group.

injection of 10^7 and 10^8 pfu RAd 7B2/27 kDa, 7B2 expression in the pituitary exhibited a strong transient peak in the first 10 d (3.5 ± 0.2 pmol/pituitary, 10^8 pfu/injection, n = 4, and 4.8 ± 0.9 pmol/pituitary, 10^7 pfu/injection, n = 4) followed by a significant decrease for a subsequent 35 d (Fig. 1A). Similar effects were measured after injection of RAd 7B2/21 kDa.

A time course of 7B2 expression in WT mice showed that after intrapituitary injection of 5×10^6 pfu of RAd 7B2/27 kDa, 7B2 content in the pituitary was three times higher (2.1 ± 0.3 pmol/pituitary, n = 4) on the 14th day after injection than the level detected in untreated controls (0.73 ± 0.03 pmol/pituitary, n = 4) (Fig. 1B). This figure also shows that intrapituitary injection of higher doses of RAd 7B2/27 kDa than 10^6 pfu/injection produces unstable transgenic expression of 7B2.

We observed relatively the same dose effect on transgene expression after delivery of RAd β gal as a control adenoviral vector (Fig. 2, A and B). The expression of β gal was detected

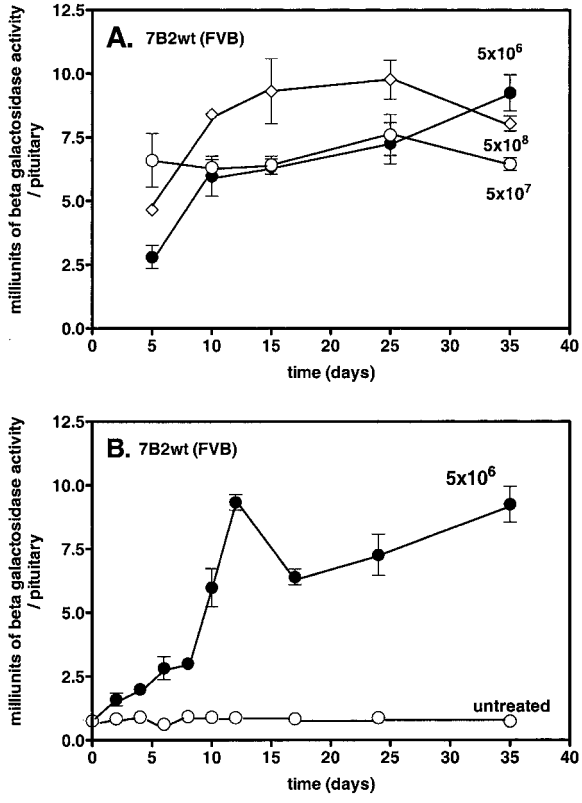

FIG. 2. Time course of β gal transgene expression in FVB mice. Transgene expression of pituitary β gal was measured as β gal activity following intrapituitary delivery of control adenoviral vector (RAd β gal) at 5×10^7 and 5×10^8 pfu/injection (A) or 5×10^6 pfu/injection (B). Points are expressed as the mean ± SD in four to five mice per group. A, *Open diamonds*, animals injected with RAd β gal 5×10^8 pfu/injection; *open circles*, 5×10^7 pfu/injection; and *filled circles*, 5×10^6 pfu/injection. B, *Open circles*, untreated control animals; and *filled circles*, animals treated with RAd β gal (5×10^6 pfu/injection).

by measuring enzymatic activity in pituitary homogenates, quantitated as milliunits/pituitary of β gal.

These data confirmed our expectation that lower doses of RAd 7B2/27 kDa exhibit more stable expression of the 7B2 transgene than do higher doses, possibly because of diminished production of intracranial inflammation.

Transgenic expression of 7B2 in pituitaries of 7B2 null and WT mice

We used groups of 7B2 null and WT mice (n = 6) at 3 wk of age derived from crosses of $7B2^{het/het}$ parents. The 7B2 transgene expression was detected by 7B2 RIA of pituitary extracts. After intrapituitary delivery of RAd 7B2/27 kDa (5×10^6 pfu/injection), the 7B2 null group showed significant 7B2 transgene expression at 14 d post injection ($P < 0.0001$, compared with control animals of the same genotype injected with RAd β gal) (Fig. 3A). Wild-type mice showed a significant ($P < 0.05$) decrease in 7B2 in pituitary (Fig. 3B). In 7B2 null mice, we detected a 3-fold increased level (154 ± 0.2 fmol/pituitary) of 7B2 over the control group injected with a control adenoviral vector (47 ± 6.4 fmol/pituitary). The low level of 7B2 in the null mice most likely represents an interference artifact in the RIA; given the relatively low total levels of 7B2, it was not possible to dilute the reconstituted extracts to eliminate this artifact. We detected an increased level of 7B2 in the plasma of 7B2 WT and null mice 14 d after injection of RAd 7B2/27 kDa (Fig. 3C). We did not detect a significant difference in the plasma levels of 7B2 in FVB mice among the various experimental groups (data not shown). Thus, 7B2 can be efficiently expressed by intrapituitary injection of adenovirus in 7B2 null mice. In WT 129/Sv mice overexpression of 7B2 appears to cause increased secretion of this molecule into the bloodstream.

Intrapituitary administration of RAd 7B2/27 kDa induces a slight increase in PC2 activity in 7B2 null mice

Intrapituitary delivery of 7B2-encoding adenovirus did not produce a detectable increase in PC2 activity in the pituitary extracts of 7B2 WT mice (7.9 ± 2.1 pmol AMC/2 h; n = 4; killed 2 wk after adenovirus injection), compared with untreated animals (10 ± 1.3 pmol AMC/2 h; n = 3) and a group treated with control adenoviral vector (9.4 ± 1.9 pmol AMC/2 h; n = 3) (Fig. 4A). Transgenic expression of 7B2 increased PC2 activity in pituitaries obtained from adenovirus-treated 7B2 null mice (1.39 ± 0.14 pmol AMC/2 h; n = 6; $P < 0.0001$), compared with the untreated group (0.064 ± 0.002 pmol AMC/2 h; n = 3) or the group treated with control adenoviral vector (0.064 ± 0.005 pmol AMC/2 h; n = 3) (Fig. 4B). PC2 activity in pituitary extracts obtained from 7B2 nulls treated with 7B2 adenovirus represented about 14% of the activity measured in untreated 7B2 WT mice.

7B2 transgene expression decreases pituitary and plasma ACTH levels in 7B2 nulls

Fourteen days after intrapituitary injection of the adenoviral vector encoding 27 kDa 7B2, pituitary levels (Fig. 5A) and plasma levels (Fig. 5C) of intact ACTH $_{1–39}$ in 7B2 null mice were significantly decreased, to 1.2 ± 0.06 μg/pituitary (n = 8; $P < 0.0001$) and 0.56 ± 0.33 ng/ml of plasma (n = 5; $P < 0.05$), compared with the level of ACTH in mice of the same genotype injected with the β gal control vector (3.2 ± 0.2 μg/pituitary, n = 7 and 2.7 ± 0.5 ng/ml plasma, n = 4). We did not detect significant changes in the levels of ACTH in the pituitaries of 7B2 WT mice (Fig. 4B) but did observe increased plasma ACTH ($P < 0.0001$) (Fig. 4D) after delivery of the 7B2/27 kDa adenovirus, compared with mice injected with RAd β gal and untreated control mice.

We concluded that adenoviral expression of 7B2 is capable of reducing pituitary hypersecretion of ACTH in the 7B2 null.

Administration of the 7B2 adenovirus suppresses corticosterone and increases blood glucose in 7B2 nulls

A significant decrease in serum corticosterone of the 7B2 null was detected after delivery of adenoviral vector encoding 27 kDa 7B2 (Fig. 6A). This effect persists for almost 1 wk, at which time the concentration of corticosterone in serum decreased by 50% (Fig. 6A). A significant decrease in the level of serum corticosterone was also evident in WT mice injected with adenovirus encoding 7B2 (Fig. 6B), despite their increased circulating ACTH levels (Fig. 5). These data indicate differential control of the hypothalamic-pituitary-adrenal

(HPA) axis in WT and null animals following adenoviral injection, most likely owing to the presence of functional corticotrophs in the anterior lobe of WT but not null mice.

In 7B2 null mice, adenoviral administration of 27 kDa 7B2 increased blood glucose, compared with control animals injected with RAd β gal. The peak of glucose detected in 7B2 nulls was 5 d after delivery, significantly increased compared with the level detected in mice of the same genotype injected with control adenoviral vector (Fig. 7A). In 7B2 WT mice of the same age, the blood glucose level showed a tendency to increase 5–14 d after delivery; a slight persistent hyperglycemia was observed in this group (Fig. 7B).

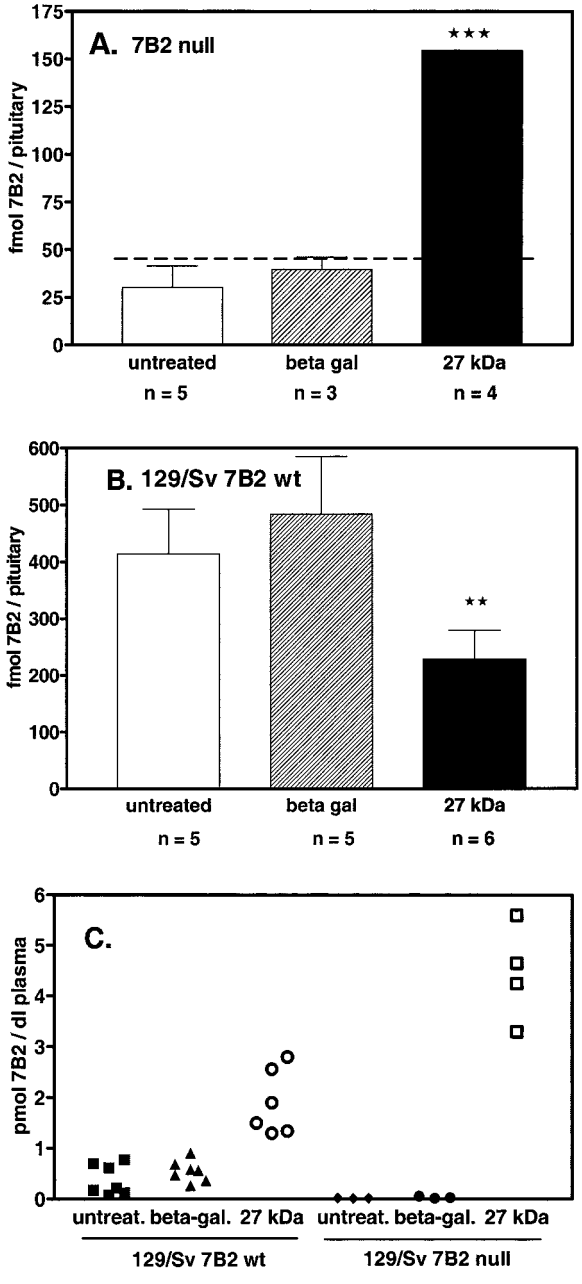

FIG. 3. 7B2 transgene expression in pituitary and plasma of 7B2 null and WT mice. Quantitation of immunoreactive 7B2 in pituitary glands of 7B2 null mice (A) and 129/SV 7B2 WT (B) and plasma levels (C) 14 d after intrapituitary delivery of RAd 7B2/27 kDa or RAd β gal (5×10^6 pfu/injection). Levels for treated groups are represented as the increase over the level in the untreated control. Results are expressed as the mean ± SD; ($P < 0.0001$ by t test) in the group of animals injected with RAd 7B2/27 kDa *vs.* the value in the group of animals injected with the control β gal RAd. C, *Filled squares*, untreated 7B2 WT animals; *filled triangles*, WT mice treated with β gal adenoviral vector; *open circles*, WT mice treated with 27 kDa adenoviral vector; *filled diamonds*, untreated 7B2 null mice; *filled circles*, null mice treated with RAd β gal; *open squares*, null mice treated with RAd 7B2/27 kDa. The *dashed line* shows the level of the acid-tissue interference artifact in the 7B2 RIA; given the relatively low total levels of 7B2 in the 7B2 null and controls, it was not possible to dilute the reconstituted extracts to eliminate this artifact.

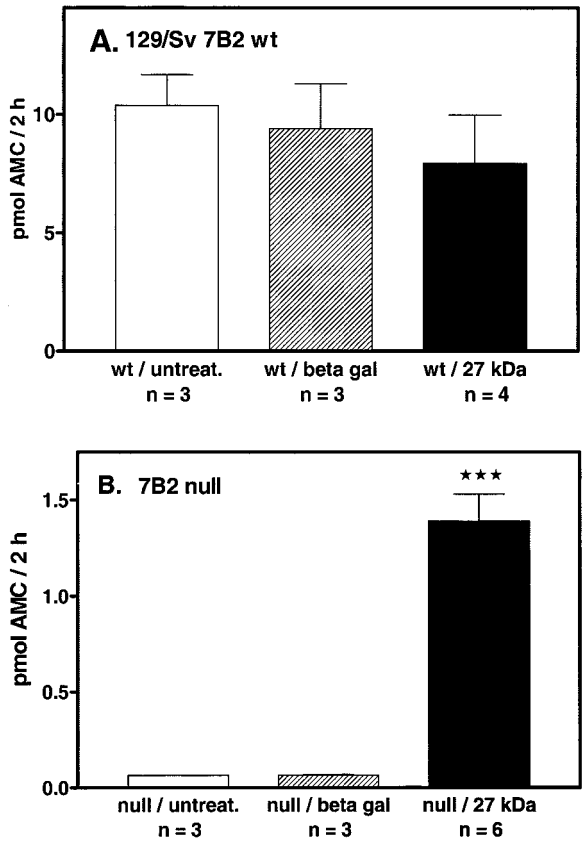

FIG. 4. Intrapituitary delivery of 7B2-encoding adenovirus increases PC2 enzyme activity in 7B2 null mice. A, PC2 enzyme assay in pituitary homogenates of 7B2 WT and null mice; B, untreated mice and mice treated with RAd7B2 and control adenoviral vector. Enzymatic activity of PC2 was assayed against a fluorogenic peptide substrate (described in *Materials and Methods*). Specificity of the PC2 assay was verified by assessing extent of inhibition using the CT peptide 1–31, a specific PC2 inhibitor. The results represent mean ± SD, n = 3–6 per group. ***, $P < 0.0001$. The PC2 activity in the untreated null mice or mice treated with control vector was not significantly different from reactions lacking tissue.

Transgenic expression of 7B2 induces an increase in circulating αMSH in 7B2 null mice

We observed a significantly elevated level of circulating αMSH (53 ± 5.6 pmol/liter) in 7B2 null mice injected with 7B2-encoding adenoviral vector, compared with animals injected with control adenoviral vector (1.1 ± 0.6 pmol/liter)

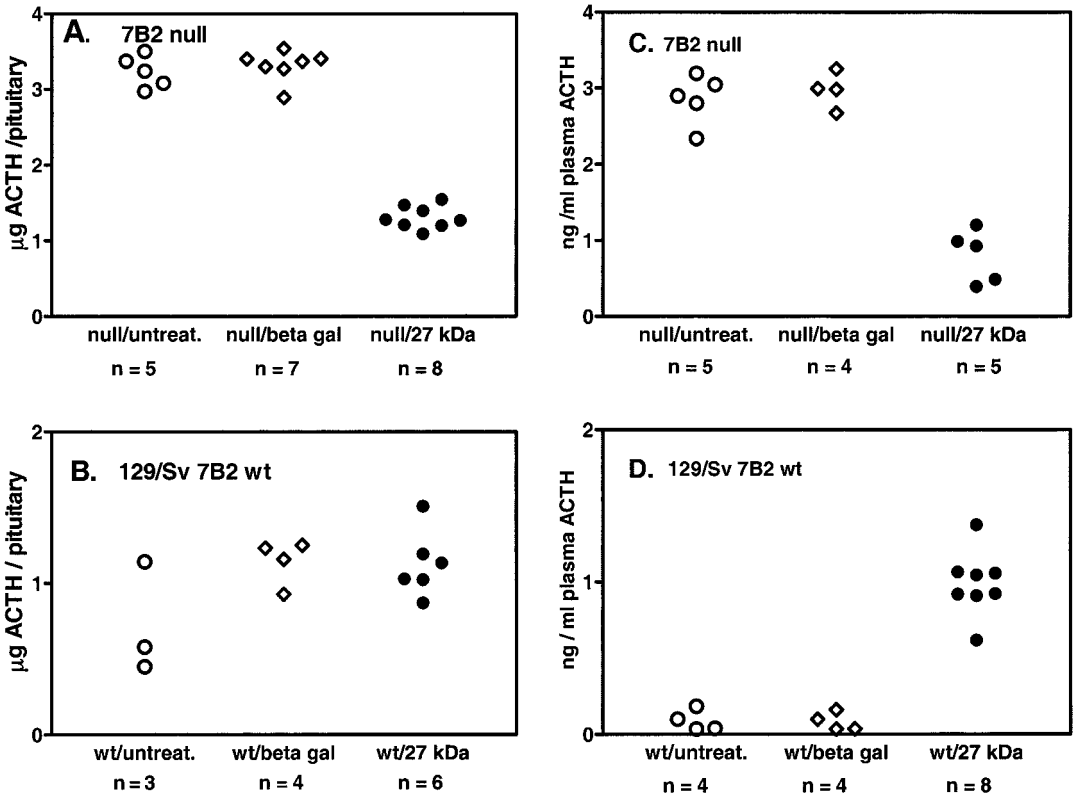

FIG. 5. 7B2 transgene expression lowers the amount of intact ACTH in 7B2 nulls. Effect of transgene expression on level of pituitary ACTH of 129/Sv 7B2 null (A) in plasma (C) and pituitary ACTH in 129/Sv 7B2 WT mice (B) and in plasma (D) 14 d after intrapituitary delivery of RAd 7B2/27 kDa and RAd β gal (5×10^6 pfu/injection). Statistical differences reached P less than 0.0001 between the group of 7B2 null mice injected with RAd 7B2/27 kDa *vs.* both control groups and between the group of 7B2 WT mice injected with adenoviral vector 7B2/27 kDa *vs.* both control groups.

or the untreated control group ($P < 0.0001$). The same effect was observed in 7B2 WT mice; treatment with 7B2-encoding virus resulted in an almost 3-fold elevated level of circulating αMSH (226 ± 8.3 pmol/liter; n = 6), compared with mice treated with control adenoviral vector (87 ± 14 pmol/liter; n = 6) or the untreated group ($P < 0.0001$) (Fig. 8, A and B). For both groups, however, circulating αMSH concentrations were less than 0.01% of circulating ACTH levels.

Expression of the 7B2 transgene in 7B2 nulls prolongs survival times

Compared with the life span in control mice injected with RAd β gal (n = 5) and in the untreated animals (n = 6), 7B2 nulls injected with a single low dose of RAd 7B2/27 kDa (n = 6) showed slightly prolonged survival (Fig. 9) ($P < 0.0001$ *vs.* controls). However, a single injection of RAd 7B2/27 kDa (5×10^6 pfu) is clearly not sufficient to rescue the lethal phenotype of 7B2 nulls because all animals died before 6 wk.

Collectively our results indicate that even a single dose of 27 kDa 7B2-encoding adenovirus is able to reduce hypercorticosteronism in 7B2 nulls 14 d after delivery. This decrease in circulating corticosterone in 7B2 null mice is associated with significant reductions in circulating and pituitary ACTH and increased circulating αMSH and glucose, with the end effect of slight but significant prolongation of life span.

Discussion

Subtilisin-like endoproteinases are involved in the processing of many prohormones in the secretory pathway (4, 20). The neuroendocrine protein 7B2 is required for the maturation of proPC2 to a form capable of generating active PC2 (4, 10); however, 7B2 may possess additional functions because it is found in nonPC2-expressing cells (8) and circulates in the blood (reviewed in Ref. 10).

7B2 null mice lack PC2 activity and exhibit multiple endocrine and metabolic pathologies (13). Most importantly, 7B2 null mice develop a Cushing's-like disease in the form of pituitary-dependent hyperadrenocorticosteronism, hypoglycemia, hyperproinsulinemia, and adrenal hypertrophy; animals die between 4 and 5 wk. With the goal of restoring functional 7B2 in the pituitary of 7B2 null mice, potentially promoting survival through the critical prepubertal and pubertal periods, we injected mice into the pituitary with a recombinant adenoviral vector encoding 7B2.

Transgenic expression of 7B2

Our initial time course experiment established that 10^6 pfu of adenovirus per injection yielded optimal expression of β gal and 7B2. 7B2 expression slowly and continuously increased, confirming previous results that a single dose of adenovirus is capable of producing detectable levels of transgene expression (21). Higher doses than 10^6 pfu/injection

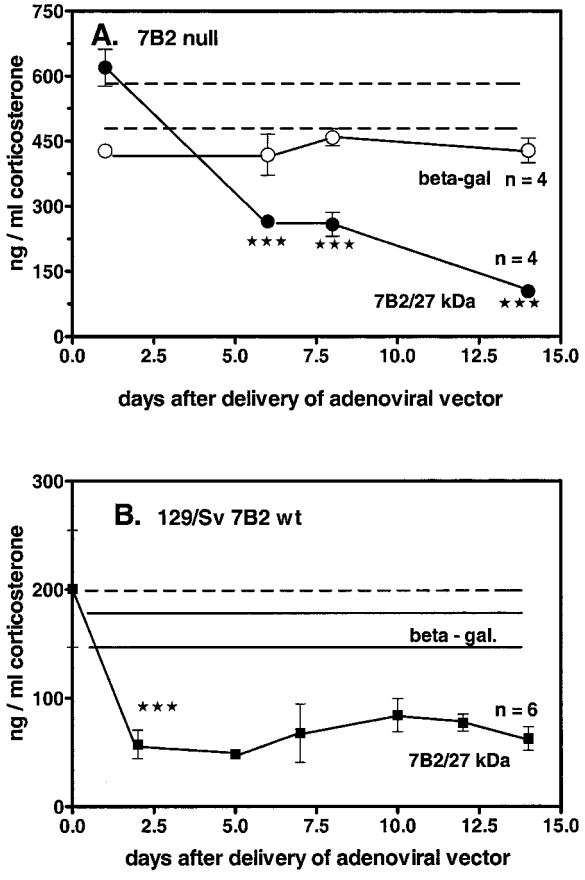

FIG. 6. The 7B2 transgene lowers serum corticosterone in 129/Sv 7B2 null and 7B2 WT mice following intrapituitary delivery of recombinant adenoviral vectors. RAds encoding 7B2/27 kDa or β gal (as a control adenoviral vector) were injected. Levels of corticosterone in the 7B2 null control untreated group were 540 ± 61 ng/ml (n = 9), and the level in the 7B2 WT control group was 140 ± 35 ng/ml (n = 7) (==), shown as a *broken double line* in Fig. 5, A and B. *Filled circles*, Animals treated with RAd β gal; 5×10^6 pfu/injection; *open circles*, animals treated with RAd 7B2/27 kDa; 5×10^6 pfu/injection.

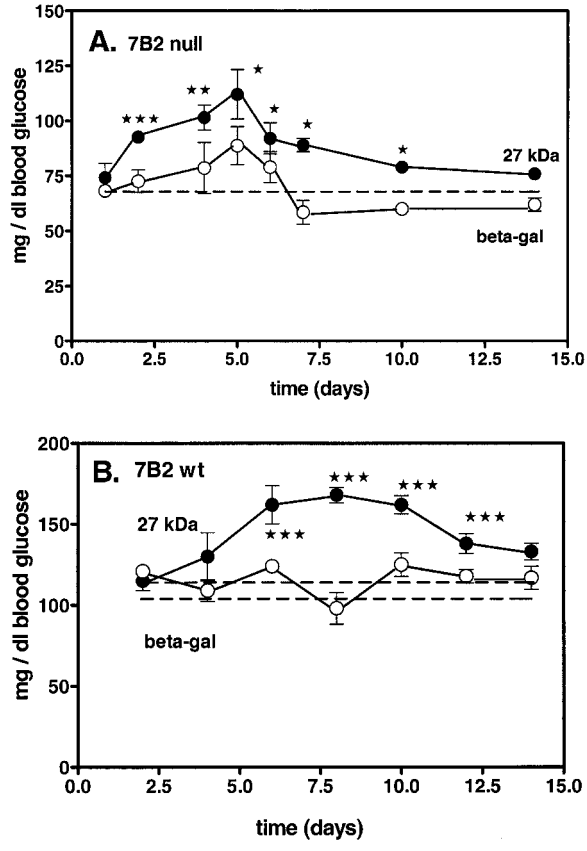

FIG. 7. Increase in blood glucose after delivery of RAd 7B2/27 kDa. Blood glucose concentrations (mg/dl) were measured at specific times after delivery of adenoviral vectors (5×10^6 pfu/injection) in 7B2 null mice (A) and 7B2 WT mice (B). The blood glucose level in the untreated 7B2 null control group was 70.4 ± 2.7 mg/dl, and 7B2 WT levels were 110 ± 9.9 mg/dl; this normal value is depicted as a *broken double line* (==). *Open circles*, Animals treated with RAd β gal; *filled circles*, animals treated with RAd7B2/27 kDa. NS, Nonsignificant differences between groups; ***, Significant differences *vs.* the group injected with RAd β gal and *vs.* the untreated control group ($P < 0.0001$).

exhibited a strong but highly transient increase in 7B2 expression. Ten days after intrapituitary delivery of adenoviral vectors at 10^7 or 10^8 pfu/injection a peak in transgene expression was observed, but this was followed by a sudden and rapid decrease. In contrast, doses of 10^6 pfu/injection exhibited a tendency to steadily increase 7B2 expression until 35 d after intrapituitary delivery. Previous studies with other adenovirus vectors used at high titers have been shown to be ineffective because of the presumed development of neutralizing antibodies in the host (19); however, multiple applications of adenoviral vectors have resulted in greater therapeutic benefits in larger animals (19).

7B2 null mice exhibit decreased pituitary and plasma ACTH levels after intrapituitary delivery of 7B2-encoding adenovirus

Without treatment, pituitary-dependent hyperadrenocorticosteronism is generally progressive and death may result from complications with sustained hypercorticosteronism such as cardiovascular disease, thromboembolism, glucose intolerance, and lactic acidosis (21a). Unlike Cushing's disease in humans, in which the development of disease results from excess ACTH secretion from anterior pituitary adenomas, 7B2 nulls develop a Cushing's-like disease due to excess circulating ACTH derived from the intermediate lobe (13). In WT animals, intermediate lobe PC2 activity (in the presence of 7B2) inactivates PC1-generated ACTH by cleavage to corticotropin-like intermediate lobe peptide and αMSH. PC2 is not well expressed in the anterior lobe, and this cleavage is therefore restricted to the intermediate lobe (22, 23). Thus, 7B2 null animals possess extremely high levels of intermediate lobe ACTH, which represents the source of the excess circulating ACTH. One of the goals of this study was to learn whether this pool of ACTH in the intermediate lobe of 7B2 nulls could be reduced by transgenic expression of 7B2 in the pituitary.

7B2 nulls, although fragile to experimental manipulation, survive intrapituitary injection without mortality. These data confirm the idea that adenovirus-mediated gene transfer is a safe and effective means to test pituitary gene transfer *in vivo* in a preclinical model (14, 24, 25). Our data show that 7B2 transgene expression in 7B2 nulls significantly decreased

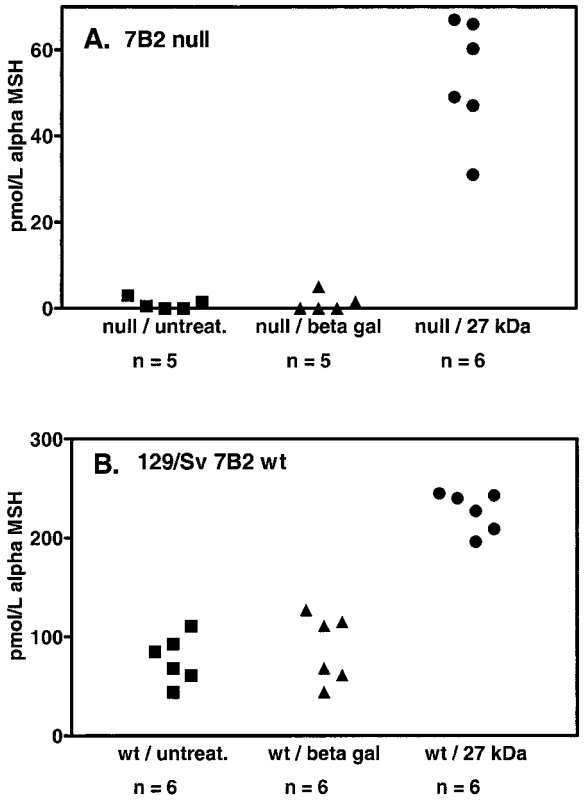

FIG. 8. Administration of 7B2-encoding adenovirus produces an increase in plasma αMSH. A, Plasma αMSH in 7B2 null mice injected with RAd 7B2/27 kDa; B, WT animals injected with Rad 7B2/27 kDa. A and B, *Filled squares,* untreated group; *filled triangles*, control group treated with the control β gal vector; *filled circles*, group treated with RAd 7B2/27 kDa. Significant differences *vs.* the group treated with control vector are marked by *** ($P < 0.0001$).

hypersecretion of intact $ACTH_{(1–39)}$ from the pituitary; this effect was not observed in WT animals. Our expectation was that 7B2 virus administration would reduce pituitary ACTH by increasing the amount of active PC2 available for cleavage of this peptide into inactive peptide products.

Plasma levels of αMSH increase after intrapituitary delivery of 7B2-encoding adenovirus

We tested for transgenic 7B2-mediated effects on proPC2 by measuring the blood levels of αMSH, a known PC2 cleavage product (26), as well as by direct measurement of pituitary PC2 activity. We noted a small elevation of PC2 activity in pituitary extracts, which represented about 14% of the activity measured in the WT pituitary. We speculate that the efficiency of viral entry into intermediate lobe cells limits the amount of 7B2-mediated restoration of PC2 activity; the fact that 7B2 levels in 7B2 adenovirus treated nulls reach only one-tenth of those in WT animals supports this idea. However, this small elevation in PC2 activity is apparently sufficient to increase circulating αMSH, presumably through a partial restoration of the ability of PC2 to cleave ACTH. Our preliminary results using primary cell cultures of 7B2 null pituitaries confirm the presence of increased αMSH after treatment with 7B2-encoding adenovirus (Hwang, J. R., and I. Lindberg, unpublished observations). Surprisingly, WT

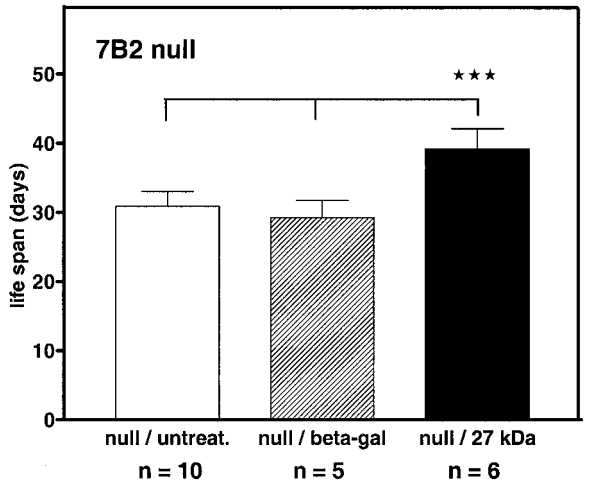

FIG. 9. Increased survival times of 7B2 null mice after delivery of RAd 7B2/27 kDa (5×10^6 pfu/injection). *Open bars*, Untreated control group of 7B2 null mice; *filled bars*, group treated with RAd 7B2/27 kDa; *gray bars*, mice treated with RAd β gal. A significant difference *vs.* the untreated control group and group injected with RAd β gal is marked by *** ($P < 0.0001$).

mice injected with 7B2 adenovirus also exhibited increased circulating αMSH but without any detectable elevation in pituitary PC2 activity. We hypothesize that either WT mice exhibited a transient increase in PC2 activity that our time frame of experimentation did not capture or, more likely, that this increase in circulating αMSH results from an unknown indirect effect of increased 7B2 expression. The role that circulating MSH might play in the endocrine homeostasis of the 7B2 null is currently unclear.

Inhibition of corticosterone hyperproduction by 7B2-encoding adenovirus

Because POMC mRNA synthesis is highly responsive to transcriptional inhibition by steroids in the anterior, but not in the intermediate, lobe of the pituitary gland, elevated circulating corticosterone will act to suppress hypothalamic secretion of CRH as well as anterior lobe synthesis and secretion of POMC products (reviewed in Ref. 27). Surprisingly, in the virus-treated 7B2 null, the reduction in the level of circulating corticosterone suppresses ACTH hypersecretion from the intermediate lobe and results in lowered circulating ACTH levels, suggesting that 7B2, by controlling the amount of corticosterone and ACTH, might play an important indirect mediatory role in the HPA axis. As intermediate lobe POMC is not thought to be under inhibitory feedback control by steroids (27), the mechanism behind a steroid-mediated drop in intermediate lobe ACTH secretion is unclear, although it appears to involve altered hypothalamic regulation of dopaminergic mechanisms (because the 7B2 null contains greatly reduced pituitary dopamine; Ref. 13a). Evidence to support this scenario consists of the observation that adrenalectomy is able to rescue the 7B2 null (13a), presumably by interrupting the ACTH/corticosterone hypersecretion loop. In view of the fact that the steroid synthesis inhibitor metyrapone does not rescue the 7B2 null (21a), other factors may be involved in adrenalectomy-mediated rescue. We hypothesized that circulating 7B2, which is in-

creased by virus administration, may contribute to a direct or indirect inhibitory effect on adrenocortical steroidogenesis. Alternatively, 7B2 expression could indirectly affect other aspects of the HPA axis (*e.g.* via effects on hypothalamic dopamine or MSH synthesis).

The different physiologies of the null and WT animals appear to result in significantly different responses to the administration of 7B2-encoding adenovirus. Although 7B2 nulls exhibit a Cushingoid endocrine pathology, with severe hypercorticosteronism, highly reduced anterior lobe corticotroph POMC synthesis (13a), and increased secretion of ACTH from the intermediate lobe, WT mice, which still contain functioning anterior pituitary corticotrophs, continue to exhibit normal feedback control of the HPA axis. In agreement with this notion, in WT animals the significant drop in circulating corticosterone normally observed after intrapituitary delivery of 7B2-encoding adenovirus was associated with an increase in circulating ACTH.

Hyperglycemic effect of 7B2-encoding adenovirus

We have previously shown that 7B2 nulls exhibit hypoglycemia; their normal level of blood glucose is approximately 75 mg/dl (13). This hypoglycemic condition in 7B2 nulls is temporarily relieved by delivery of a low dose of 7B2-encoding adenovirus to the pituitary; elevation in blood glucose persisted for about 2 wk. Control of blood sugar in the 7B2 null is complex. 7B2 null mice exhibit elevated circulating insulin-like material because of islet hypertrophy, but the major processing defect in the pancreas is most likely the inability to cleave proglucagon forms to mature glucagon, a defect shared by the PC2 null (13, 28). The hyperglycemic effect of transgenically expressed 7B2 in 7B2 nulls is not likely to arise from reestablishment of pancreatic PC2 activity, *i.e.* increased conversion of proglucagon forms to mature glucagon, because pituitary delivery of virus is unlikely to result in significant pancreatic expression. The hyperglycemic effect of 7B2-encoding virus could result either from the decrease in corticosterone or indirectly via a contribution of an unknown function of circulating 7B2 that ultimately impacts on blood sugar levels. The use of repeated low doses of adenovirus might produce a more profound effect in this regard; alternatively, a different means of delivery of recombinant adenoviral vector could be used, for example a catheter-based intravascular infusion method, to obtain longer-lasting benefits on blood glucose (29). Preliminary results in our laboratory show that after intrapituitary delivery of a very high dose of 7B2-encoding virus in 7B2 null mice, blood glucose increases to an even greater extent than in the experiments reported here (Sarac, M., and I. Lindberg, unpublished observations).

Intrapituitary delivery of 7B2 transgene in 7B2 nulls and WT mice results in an interesting distribution of transgenically expressed 7B2; a portion appears to be stored in the pituitary, and data showing an increase in circulating 7B2 indicate that another portion is released into the circulation. The role of circulating 7B2, demonstrated over 20 yr ago (reviewed in Ref. 10), is currently unknown. We speculate that circulating 7B2 may have effects on blood glucose and the HPA axis that are unrelated to effects on PC2. The facts that 7B2 is much more widely expressed than PC2 (8, 30, 31) and that PC2 nulls do not develop signs of Cushing's (Ref. 32 and our unpublished results) support the idea that 7B2 might have physiological roles unrelated to effects on PC2.

Recent data from our laboratory show that sudden and severe hypoglycemia can potentially represent a primary cause of death in 7B2 nulls (21a). The critical period of survival in 7B2 nulls is the prepubertal and early pubertal period, between 4 and 6 wk of age. Our data show that a single low dose of adenoviral vector encoding 7B2 into 7B2 nulls effected a slight but significant prolongation of life span in these animals, most likely through beneficial effects on blood sugar. Possibly more profound, or longer, 7B2 overexpression is required to effect a more complete reversal of the deleterious effects of 7B2 deprivation.

In conclusion, the present data showing alterations in glucose, corticosterone, plasma, and pituitary ACTH, and αMSH as a consequence of administration of 7B2-encoding adenovirus support the idea that adenovirally mediated gene transfer will represent an effective method to study the physiology of the neuroendocrine protein 7B2 *in vivo*.

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