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Targeted Inactivation of the Gene Encoding the Mouse α_{2C}-Adrenoceptor Homolog

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SUMMARY

α_2-Adrenergic receptors (α_2-ARs) regulate a wide range of physiological functions and are targets for clinically important antihypertensive and anesthetic agents. Three genes encoding α_2-AR subtypes have been cloned in humans and mice, but the physiological significance of each subtype has not been completely characterized. The available agonist and antagonist compounds are not sufficiently subtype selective to allow the unambiguous dissection of these receptors *in vivo*. As an alternative approach, we have used gene targeting in embryonic stem cells to disrupt the *Adra2c* gene, which encodes the α_{2C}-AR subtype in mice. *Adra2c*$^-$/*Adra2c*$^-$ animals do not express a functional α_{2C}-AR transcript, as detected by Northern blotting or reverse transcription-polymerase chain reaction analysis. In addition, these mice have markedly reduced [^{3}H]rauwolscine binding in their caudate putamen and in other brain regions normally expressing *Adra2c* binding sites. *Adra2c*$^-$/*Adra2c*$^-$ mice, however, are viable and fertile and appear grossly normal. Expression levels of *Adra2a* and *Adra2b* mRNA in brain and kidney are not altered by the *Adra2c* knockout. These data suggest that up-regulation of *Adra2a* or *Adra2b* does not compensate for the *Adra2c* deficiency and that the receptor encoded by *Adra2c* is not required for normal mouse development or for survival in a laboratory environment.

The α_2-ARs are members of the G protein-coupled receptor superfamily and mediate many of the physiological actions of the endogenous catecholamines epinephrine and norepinephrine. α_2-ARs have been implicated in a wide range of physiological processes, including the regulation of sympathetic tone, cardiovascular functions, lipid and carbohydrate metabolism, platelet aggregation, analgesia, gastrointestinal motility, maintenance of fluid and electrolyte balance by the kidneys, and modulation of neurotransmitter release from presynaptic nerve terminals (1, 2). In addition, α_2-AR agonists potently reduce anesthetic requirements in humans and animals (3–5) and profoundly influence the seizure threshold in animal models of epilepsy (6–8).

Based on radioligand binding data, Bylund *et al.* (9) originally subdivided the α_2-ARs into three pharmacological subtypes, termed α_{2A}, α_{2B}, and α_{2C}. In bovine pineal gland and in rodent tissues, a fourth pharmacological subtype, the α_{2D}, has been identified. It diverges slightly from the α_{2A} subtype in its pharmacological ligand-binding properties (10, 11); nevertheless, molecular biological evidence suggests that the α_{2D} subtype represents the rodent homolog of the α_{2A}-AR defined in humans (12, 13). The α_{2A}, α_{2B}, and α_{2C} subtypes are encoded by three different genes, located on human chromosomes 10, 2, and 4, respectively (14–17). The subsequent isolation of gene homologs from rats (*RG20*, *RNG*-α_2, and *RG10*) (10, 18–20) and mice (*Adra2a*, *Adra2b*, and *Adra2c*) (12, 21) confirmed the conservation of α_2-AR gene divergence across mammalian species. To date, no more than three subtypes have been convincingly demonstrated in any species.

A growing body of evidence supports the hypothesis that the three α_2-AR subtype proteins are functionally distinct. At the level of primary protein sequence, it is possible to identify subtype-specific features that are conserved across species, such as the length of the amino terminus and the presence or absence of consensus sites for glycosylation and phosphorylation. Although all three α_2-ARs couple to the G_i family of heterotrimeric G proteins, they do exhibit differences in coupling to other G proteins (i.e., G_s) when expressed in cultured

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ABBREVIATIONS: α_2-AR, α_2-adrenergic receptor; pBS, pBluescript II SK−; ES cells, embryonic stem cells; PGK, phosphoglycerate kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase(s); TM, transmembrane domain; *Adra2c*, murine of α_2-AR; pA, polyadenylation sequence.

transfected cells (22, 23). The subtypes also differ in their steady state subcellular localization and in their trafficking in response to agonist stimulation (24). Finally, individual α_2-AR subtypes have unique patterns of tissue distribution *in vivo* (25–30). These data argue against significant functional redundancy for these proteins.

Certain synthetic agonists and antagonists can differentiate among α_2-AR subtypes *in vitro*, but these compounds have not been particularly useful for characterizing subtype-specific functions *in vivo*, for several reasons. First, complete pharmacological selectivity for a single α_2-AR subtype cannot be achieved with currently available α_2-AR agonists or antagonists. This is particularly true *in vivo*, where tissue levels of drugs are often difficult to predict. Second, even in cases where a drug is somewhat selective for an α_2-AR subtype, its interactions with other receptor classes (e.g., α_1-ARs or dopamine receptors) complicate the interpretation of the results. Third, α_2-AR subtypes are expressed at very low levels in some tissues, making it difficult to localize individual subtypes by using radioligands or by *in situ* hybridization. Thus, although remarkable advances have been made in our understanding of α_2-AR function at the molecular and cellular levels, relatively little progress has been made in assigning functional roles to specific α_2-AR subtypes *in vivo*, and the physiological significance of multiple α_2-AR genes remains an important unresolved question.

An alternative and complementary approach to probing the physiological significance of any gene product *in vivo* is based on gene targeting in mouse ES cells (31, 32). So-called gene knockouts have been generated for a variety of tyrosine kinase and steroid hormone receptors and, recently, mice carrying a serotonin receptor knockout were reported to exhibit increased aggression (33). Here we report the production and preliminary characterization of mice lacking a functional *Adra2c* gene encoding the α_{2C}-AR subtype, which represents the murine homolog of the α_2-AR located on human chromosome 4.

Materials and Methods

Construction of the *Adra2c* gene-targeting vector. A 6.5-kb *Eco*RI fragment from phage EMBL3, containing the murine α_{2C} gene (*Adra2c*; previously termed Mα_2-4H) (12), was subcloned into pBS and designated pBS-*Adra2c*. *Adra2c* contains no introns within its coding sequence. pBS-*Adra2c-NB2* was prepared by removing genomic sequences 5′ to the *Nco*I site in pBS-*Adra2c*. A 3.5-kb *Sal*I/*Not*I fragment derived from pBS-*Adra2c*, containing sequences 5′ to the receptor coding sequence, was subcloned into pBS to create pBS-*sub5′-2c*. pBS-*trunc-2c* was created by subcloning a 6.1-kb *Sal*I/*Not*I fragment from pBS-*Adra2c* into pBS and converting the unique *Mlu*I site in the receptor coding sequence to a *Cla*I site via an oligonucleotide adapter. pBS-*trunc-2c* does not contain genomic sequences 3′ to the *Bam*HI site in pBS-*Adra2c*. pGK-*NeoBpa*/*Cla* was constructed by converting the 3′ *Not*I site in pGK-*NeoBpa* (34) to a *Cla*I site with an oligonucleotide adapter. A *Cla*I cassette containing the PGK promoter, neomycin resistance gene, and bovine growth hormone polyadenylation sequence from pGK-*NeoBpa*/*Cla* was inserted into a unique *Mlu*I site within the *Adra2c* coding sequence from pBS-*trunc-2c*, giving pBS-*2c-Neo*. The *Neo* sequence was inserted in the same orientation as the *Adra2c* open reading frame, placing a premature termination codon immediately after TM5. This truncated receptor lacks critical structural determinants required for ligand binding (TM6–7) and G protein coupling (cytoplasmic loop 3) and should be nonfunctional. To construct the final *Adra2c* gene-targeting vector, pBS-*Adra2c-KO* (Fig. 1a), a three-fragment ligation was performed with the following fragments: a 5.5-kb *Xho*I/*Bam*HI vector fragment derived from pPNT (34), a 3.5-kb *Xho*I/*Not*I fragment from pBS-*sub5′-2c*, and a 4.2-kb *Not*I/*Bam*HI fragment from pBS-*2c-Neo*. pBS-*Adra2c-KO* contains 6 kb of isogenic sequence.

Southern and Northern blotting and recombinant DNA probes. Southern analysis was carried out as described previously (12). Poly(A)$^+$ RNA was prepared from mouse brain and kidney with the FASTRACK kit (Invitrogen, San Diego, CA) and was analyzed by standard Northern blotting methods (35).

A variety of DNA probes were isolated, labeled with ^{32}P by random priming, and hybridized to blots, including the following: *Adra2a*(Coding), a 1.4-kb *Nco*I/*Sph*I fragment derived from the mouse *Adra2a* coding sequence; *Adra2b*(Coding), a 1.3-kb *Nco*I/*Bam*HI fragment derived from the mouse *Adra2b* coding sequence; *Adra2c*(Front), a 0.72-kb *Nco*I/*Mlu*I fragment derived from mouse *Adra2c* coding sequence 5′ to the *Neo* insertion site in pBS-*Adra2c-KO*; *Adra2c*(Back), a 0.86-kb *Mlu*I/*Bam*HI fragment derived from mouse *Adra2c* coding sequence 3′ to the *Neo* insertion site in pBS-*Adra2c-KO*; *Adra2c*(External), a 0.39-kb *Bam*HI/*Eco*RI fragment 3′ to the *Adra2c* coding sequence [*Adra2c*(External) is composed of sequences not included in pBS-*Adra2c-KO* and, therefore, should not detect constructs that are integrated into the genome in a nontar-

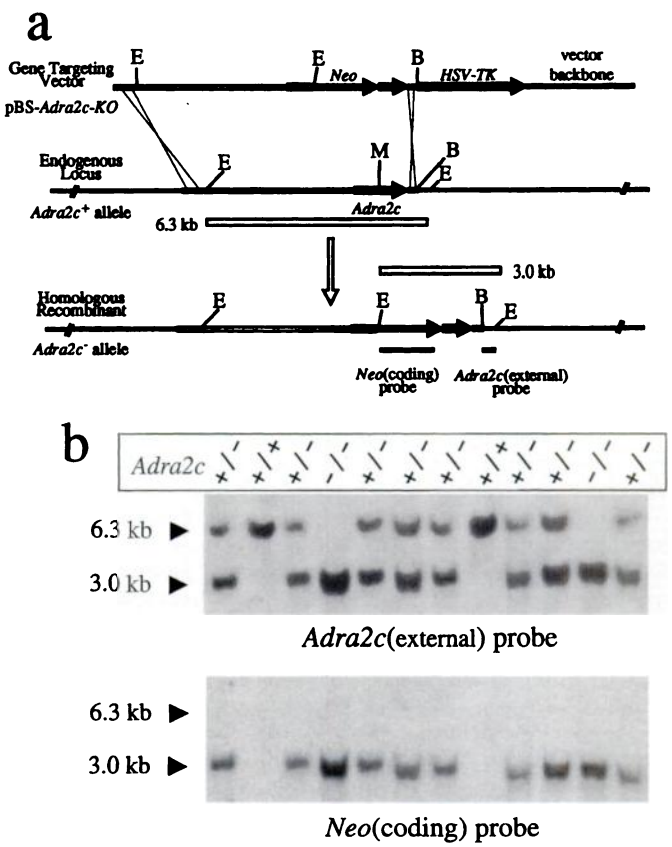

Fig. 1. Generation of mice lacking a functional *Adra2c* gene. a, Strategy for detecting homologous replacement events at the *Adra2c* locus in mouse ES cells and mice. *E*, *Eco*RI site; *B*, *Bam*HI site; *M*, *Mlu*I site; *Neo*, neomycin resistance cassette; *HSV-TK*, thymidine kinase cassette derived from herpes simplex virus; *Adra2c*, *Adra2c* coding sequence. *Black bars*, probes used for hybridization to Southern blots [*Adra2c*(External) and *Neo*(Coding)]; *white bars*, *Eco*RI fragments detected by *Adra2c*(External). b, Southern analysis of tail DNAs derived from progeny of *Adra2c*$^+$/*Adra2c*$^-$ intercrosses at weaning. Viable *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ animals were recovered from these crosses. After hybridization with *Adra2c*(External), the same blot was stripped and reprobed with *Neo* (Coding).

geted fashion]; *Adra2c*(RT), a 380-bp *SacI/MluI* fragment from pBS-*Adra2c-NB2*; *Neo*(Coding), a 0.82-kb *PstI/XbaI* fragment derived from PGK-*NeoBpa*; and GAPDH, an 1.2-kb *PstI* fragment from the mouse *GAPDH* gene.

Isolation of ES cell clones containing targeted replacement events at the *Adra2c* locus. Gene-targeting experiments in ES cells were carried out using a modification of previously published protocols (36). The R1 line of mouse ES cells used for these experiments has been described previously (37) and was generously provided by Dr. Andras Nagy (Mount Sinai Hospital, Toronto, Canada). R1 cells were cultured at 37° in 95% air/5% CO_2, on confluent monolayers of irradiated primary embryonal fibroblasts, in Dulbecco's modified Eagle's medium (University of California, San Francisco, Tissue Culture Facility, San Francisco, CA) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 1 mM sodium pyruvate (Gibco), nonessential amino acids and penicillin/streptomycin (1×; University of California, San Francisco, Cell Culture Facility), 0.1 mM β-mercaptoethanol, and 2000 units/ml leukemia inhibitory factor (ESGRO; Life Technologies, Gaithersburg, MD).

For the targeting experiments, 10^7 R1 cells were electroporated with 20 μg of pBS-*Adra2c-KO* that had been previously linearized by digestion with *Not*I. After culture for 9 days in medium containing G418 and gancyclovir, approximately 500 colonies were visible (10-fold more colonies were recovered in parallel experiments with G418 alone, suggesting enrichment for homologous recombination events). Of these colonies, 66 were picked into microtiter plates, and replicas were either frozen or expanded for DNA analysis. Approximately 2×10^5 cells from each colony were used to prepare genomic DNA, which was digested with *Eco*RI, electrophoresed through a 1% agarose gel, and transferred to a nylon membrane for Southern analysis. After hybridization with the ^{32}P-labeled *Adra2c*(External) probe, homologous recombination events could be identified by a shift in band size from 6.3 to 3.0 kb (Fig. 1a).

Production of *Adra2c*$^{\pm}$ mice from ES cell clones. The generation of chimeric mice by blastocyst injection of ES cells was carried out essentially as described by Bradley (38). R1 cells containing a targeted mutation at the *Adra2c* locus were injected into C57BL/6J blastocysts, and the resulting chimeric mice were bred to F_1 (C57BL/6J × DBA/2J) or FVB/N animals. The latter cross is a starting point in the production of congenic strains suitable for breeding to transgenic animals produced in our laboratory on an FVB/N background. Attempts to maintain the *Adra2c*-knockout mutation on an inbred 129/Sv background were not successful.

RT-PCR analysis. The strategy for detection of the wild-type *Adra2c* transcript by RT-PCR is outlined in Fig. 3a. Total RNA was prepared from whole brains of *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ mice, using standard techniques (35). For some samples, 25 μg of RNA were treated with DNase for 1 hr at 37° in a reaction containing 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 60 units of RNAsin (Promega), and 25 units of RNase-free DNase (Stratagene). This was a necessary control because the *Adra2c* gene lacks introns. Five micrograms of total RNA were reverse transcribed using a specific *Adra2c* antisense primer (α#7) from TM6 to prime cDNA synthesis [in a 20-μl reaction mixture containing 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 60 units of RNasin (Promega), 0.5 mM levels of each deoxynucleoside triphosphate, and 50 pmol of primer (5′-TAAAGAGCGGTTCTGGCA-3′), at 70° for 10 min]. After quick chilling on ice, 400 units of Superscript II reverse transcriptase (Gibco-BRL) were added and the reaction mixture was incubated for 1 hr at 42°, followed by a 10-min incubation at 95° to inactivate the enzyme. Mock cDNA synthesis reactions lacking reverse transcriptase were carried out for all samples. Two microliters of cDNA were subjected to 21 cycles of PCR using an amplification protocol described previously (39). For these experiments, the sense primer (α#2, 5′-TTCACCGTGGTAGGCAAT-3′) was located in TM2 and the antisense primer (α#1, 5′-TTTCTCGCTGAGCGTACG-3′) was located immediately following the *Mlu*I site into which the *Neo* cassette was inserted to make the *Adra2c*^{Neo} allele. PCR products were separated on 2% agarose gels, transferred to nylon membranes, and detected by hybridization with the ^{32}P-labeled *Adra2c*(RT) probe, which is internal to the primer sequences. This strategy should detect only intact, potentially functional, *Adra2c* mRNA.

Radioligand binding. Two adult mice from each group were killed by decapitation, and their brains were rapidly dissected and frozen by immersion in cold isopentane in a dry ice bath. Coronal 14-μm sections were cut on a cryostat and thaw-mounted onto gelatin-coated slides. The slides were first dried at room temperature for 2 hr and then stored at −70° with desiccant in sealed containers.

Radioligand binding assays were modified from earlier reports on the use of the α_{2C}-preferring radioligand [^{3}H]rauwolscine (80 Ci/mmol; DuPont-NEN, Dreieich, Germany) for receptor autoradiography (40). The assay conditions were optimized and validated in preliminary experiments. Incubations were carried out at room temperature in 14-ml plastic slide mailers, in 50 mM potassium phosphate buffer, pH 7.4. The incubation time was 60 min, and washes were for 20 and 40 min in the same buffer at 4°. Next, the slides were briefly dipped in cold water to remove salts and were dried under a stream of cool air. The specificity of α_2-AR binding was determined in parallel incubations supplemented with 100 μM (−)-epinephrine (Sigma Chemical Co., St. Louis, MO). In some experiments, radioligand binding to α_{2A}-ARs was masked with 100 nM oxymetazoline (Sigma).

Radiolabeled, dried tissue sections, along with autoradiographic ^{3}H-microscales (Amersham), were apposed to tritium-sensitive film (Hyperfilm ^{3}H; Amersham) for 5 weeks. Films were developed with Kodak D-19 developer, and the autoradiographic images were analyzed with a computerized image analysis system (MCID M4; Imaging Research, St. Catharines, Ontario, Canada). Using shading correction, the images were captured with a charge-coupled device video camera (Hamamatsu C3077; Hamamatsu Photonics, Hamamatsu City, Japan) and digitized into an array of 640 × 480 pixels with a density range of 0–255. A standard curve was generated by measuring and plotting the optical densities of the images of the plastic microscales versus their radioactivities. Areas of interest were identified and traced, and their optical densities were measured and converted to radioactivity values by interpolation. Values for nonspecific binding were subtracted from total binding values. Nonspecific binding, determined with 100 μM (−)-epinephrine, always represented <10% of total binding. The results for specific binding are expressed as femtomoles of radioligand bound per milligram of tissue weight (estimated from the area and thickness of the tissue sections). Histological control sections were stained with hematoxylin/eosin or cresyl violet, according to standard procedures, or were stained for acetylcholinesterase by the *S*-acetylthiocholine method (41). No statistical analysis was carried out, because only two animals from each group were analyzed.

In addition, seven 5-week-old mice (of either sex) from each group were killed for conventional radioligand binding assays. Their striata were rapidly dissected, pooled, and homogenized in 10 volumes of buffer, and crude membrane fractions were prepared by centrifugation. The membranes were washed once and used for saturation binding assays using the non-α_2 subtype-selective radioligand [^{3}H]RX821002 (0.1–4 nM) or the α_{2C}-preferring radioligand [^{3}H]rauwolscine (0.1–4 nM; with 100 nM oxymetazoline to block α_{2A}-ARs) (42). (−)-Epinephrine (100 μM) was used to determine the specificity of binding. Saturation isotherm data were analyzed by a nonlinear, least-squares, curve-fitting technique, using GraphPAD software (GraphPAD Software Inc., San Diego, CA).

Results

Targeted inactivation of the murine *Adra2c* gene in ES cells and mice. The technical aspects of gene targeting in ES cells have been reviewed in detail elsewhere (36, 43).

The strategy for inactivating one copy of the *Adra2c* gene in ES cells is outlined in Fig. 1a. The *Adra2c* gene-targeting vector, pBS-*Adra2c-KO*, was transfected into R1 ES cells, which were then grown under selection conditions as described in Materials and Methods. Of the 66 colonies assayed by Southern blotting, 24 (36%) represented homologous replacement events, giving an absolute targeting frequency at the *Adra2c* locus of approximately $1/5.5 \times 10^4$ transfected cells. Ten homologous recombinant ES cell clones were injected into C57BL/6J blastocysts, and six chimeric animals were produced. Two chimeras derived from independent ES cell lines (C4-H2 and C4-E7) transmitted the *Adra2c*-knockout allele to their offspring.

Viability of *Adra2c*$^-$/*Adra2c*$^-$ mice. Animals derived from the ES cell line C4-H2 and heterozygous for the *Adra2c*-knockout allele (*Adra2c*$^-$) were intercrossed, and their progeny were analyzed by Southern blot analysis of tail DNA, as shown in Fig. 1b. From 247 intercross progeny studied at weaning, 60 *Adra2c*$^+$/*Adra2c*$^+$, 133 *Adra2c*$^+$/*Adra2c*$^-$, and 54 *Adra2c*$^-$/*Adra2c*$^-$ animals were obtained. These proportions are consistent with Mendelian expectations ($\chi^2 = 1.75$; $p > 0.2$). Only a single 3.0-kb band corresponding to the *Adra2c*$^-$ allele was detected with the *Neo*(Coding) probe in these animals (Fig. 1b), confirming that they do not carry additional copies of pBS-*Adra2c-KO* integrated by nonhomologous recombination elsewhere in the genome. Similar results were obtained in crosses of animals derived from the ES cell line C4-E7.

The results from *Adra2c*$^+$/*Adra2c*$^-$ intercrosses clearly demonstrated that mice of the *Adra2c*$^-$/*Adra2c*$^-$ genotype did not exhibit elevated embryonic or perinatal mortality. In addition, adult *Adra2c*$^-$/*Adra2c*$^-$ mice appeared grossly normal and were fertile, with *Adra2c*$^-$/*Adra2c*$^-$ × *Adra2c*$^-$/*Adra2c*$^-$ crosses producing litters of normal size (8.3 ± 0.7 pups/litter; seven litters).

Analysis of *Adra2c* mRNA expression in *Adra2c*-mutant mice. The mutation engineered at the *Adra2c* locus does not delete the entire coding sequence of the receptor. Rather, it interrupts the coding sequence with a *PGK-Neo* cassette, placing translational stop codons in all three frames and a polyadenylation signal 5′ to the third intracellular loop of the receptor. To determine the effect of the *PGK-Neo* insertion on *Adra2c* transcription and RNA processing, we analyzed poly(A)$^+$ RNA by Northern blot hybridization with probes from different portions of the *Adra2c* gene [*Adra2c*(Front) and *Adra2c*(Back)] (Fig. 2) and a probe from the *PGK-Neo* coding sequence [*Neo*(Coding)]. For these experiments, RNA was prepared from whole brain and kidney, because previous studies in rats indicated that rat homologs of the *Adra2a* and *Adra2c* genes (*RG20* and *RG10*, respectively) are expressed at high levels in brain, whereas the rat homolog of the *Adra2b* gene (*RNG-*α_2) is expressed at high levels in kidney (25, 27–29, 44).

The *Adra2c*(Front) and *Adra2c*(Back) probes detected the nonmutant 2.9-kb mRNA (Fig. 2a, transcript A) in *Adra2c*$^+$/*Adra2c*$^+$ and *Adra2c*$^+$/*Adra2c*$^-$ mice but not in *Adra2c*$^-$/*Adra2c*$^-$ mice. The *Adra2c*(Front) probe also detected a 3.3-kb mRNA specific for the *Adra2c*$^-$ allele in *Adra2c*$^+$/*Adra2c*$^-$ and *Adra2c*$^-$/*Adra2c*$^-$ mice (Fig. 2a, transcript B) but not in *Adra2c*$^+$/*Adra2c*$^+$ mice. This 3.3-kb mRNA was also detected by the *Neo*(Coding) probe (Fig. 2) and, therefore, most likely represents a transcript that begins at the

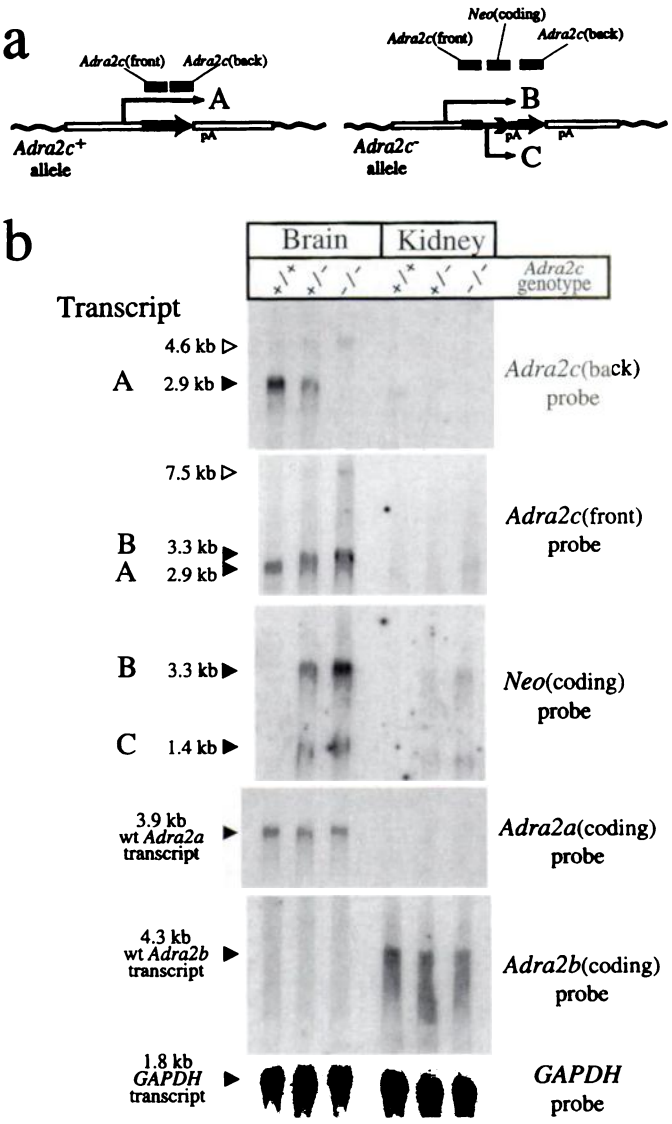

Fig. 2. Northern analysis of poly(A)$^+$ RNA isolated from *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ mice. a, Diagram of mRNAs transcribed from the *Adra2c*$^+$ and *Adra2c*$^-$ alleles. *Cross-hatched arrows*, *Adra2c* coding sequence; *thick black arrow*, location of the *PGK-Neo* cassette in the *Adra2c*$^-$ allele; *thin black arrows*, predicted mRNA transcripts, which were assigned identifying letters (A–C). b, Northern blot of whole brain or kidney poly(A)$^+$ RNA sequentially hybridized to various ^{32}P-labeled probes (described in Materials and Methods). The blot was stripped between hybridizations. Each lane represents poly(A)$^+$ RNA pooled from two mice. The identity (A–C) and approximate molecular weight of each transcript are shown to the *left of the blot*. *wt*, wild-type. *Open arrowheads*, high molecular weight transcripts that cannot encode functional receptors but whose identity has not been characterized in detail (see Results).

Adra2c transcription initiation site and ends at the *PGK-Neo* polyadenylation site. A 1.4-kb mRNA, which was detected only by the *Neo*(Coding) probe and not by either *Adra2c* probe (Fig. 2), was found in *Adra2c*$^+$/*Adra2c*$^-$ and *Adra2c*$^-$/*Adra2c*$^-$ mice and, therefore, most likely represents a transcript initiating at the *PGK-Neo* initiation site and terminating at the *PGK-Neo* polyadenylation site (Fig. 2a, transcript C). Finally, two large transcripts, of 7.5 and 4.6 kb, specific for the *Adra2c*$^-$ allele (Fig. 2b, *open arrowheads*) were detected at low levels. The 7.5-kb mRNA was detected only by the *Adra2c*(Front) probe, the 4.6-kb mRNA was detected only

by the *Adra2c*(Back) probe, and neither was detected by the *Neo*(Coding) probe. The origin of these transcripts is not yet known; however, because the 7.5-kb mRNA was not detected by the *Adra2c*(Back) probe and the 4.6-kb mRNA was not detected by the *Adra2c*(Front) probe, neither transcript contains sufficient information to encode a functional receptor protein.

To investigate the possibility that cryptic splicing events might produce a functional *Adra2c* transcript at levels too low for detection by Northern blotting, we used RT-PCR to study brain mRNA from *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ mice (Fig. 3). Because the *Adra2c* coding sequence contains no introns, some samples were treated first with RNase-free DNase to remove small amounts of contaminating DNA. A 551-bp product representing the intact *Adra2c* mRNA was detected only in samples from *Adra2c*$^+$/*Adra2c*$^+$ and *Adra2c*$^+$/*Adra2c*$^-$ mice, confirming that the *Adra2c*$^-$ allele cannot encode a functional receptor transcript. An additional, smaller (256 bp), band was detected for all genotypes when reverse transcriptase was included in the cDNA synthesis reaction. Sequence analysis of this 256-bp fragment revealed that it was a product derived from mispriming of primer α#7, at position 429 in the *Adra2c* coding sequence, during cDNA synthesis.

Analysis of α_2-AR binding sites in brain by radioligand binding. As an alternative approach to the study of α_{2C}-AR expression in *Adra2c*-mutant mice, we examined [^{3}H]rauwolscine binding to brain sections, by autoradiography and quantitative image analysis. Previous *in situ* hybridization studies showed that RNAs encoding the rat α_{2A}- and α_{2C}-AR subtypes predominate in brain (25, 28). The murine α_{2A}-AR has a low affinity for rauwolscine and a high affinity for oxymetazoline, compared with the α_{2C}-AR. Therefore, it is possible to preferentially examine α_{2C}-AR expression by performing binding studies with 0.8 nM [^{3}H]rauwolscine in the presence of 100 nM oxymetazoline. Binding results were obtained from two independent experiments on brain sections from two *Adra2c*$^-$/*Adra2c*$^-$ mice, two *Adra2c*$^+$/*Adra2c*$^-$ mice, and one *Adra2c*$^+$/*Adra2c*$^+$ mouse. Representative autoradiograms are shown in Fig. 4, and the quantitation results are summarized in Table 1. In *Adra2c*$^+$/*Adra2c*$^+$ mice,

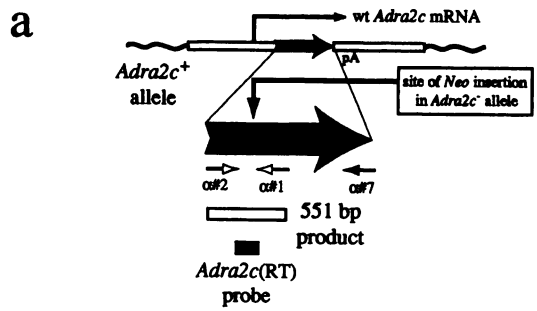

Fig. 3. Analysis of *Adra2c* expression by RT-PCR. a, Diagram of the strategy for detecting intact *Adra2c* RNA by RT-PCR. *Black arrow*, cDNA synthesis primer (α#7); *white arrows*, PCR amplification primers (α#1 and α#2). *wt*, wild-type. The size of the predicted product (551 bp) and the position of the *Adra2c*(RT) probe are also shown. b, Southern blot of RT-PCR products amplified from total brain RNA from *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ mice. Products were visualized by hybridization with the ^{32}P-labeled *Adra2c*(RT) probe. *Cross-hatched arrow*, *Adra2c* coding sequence.

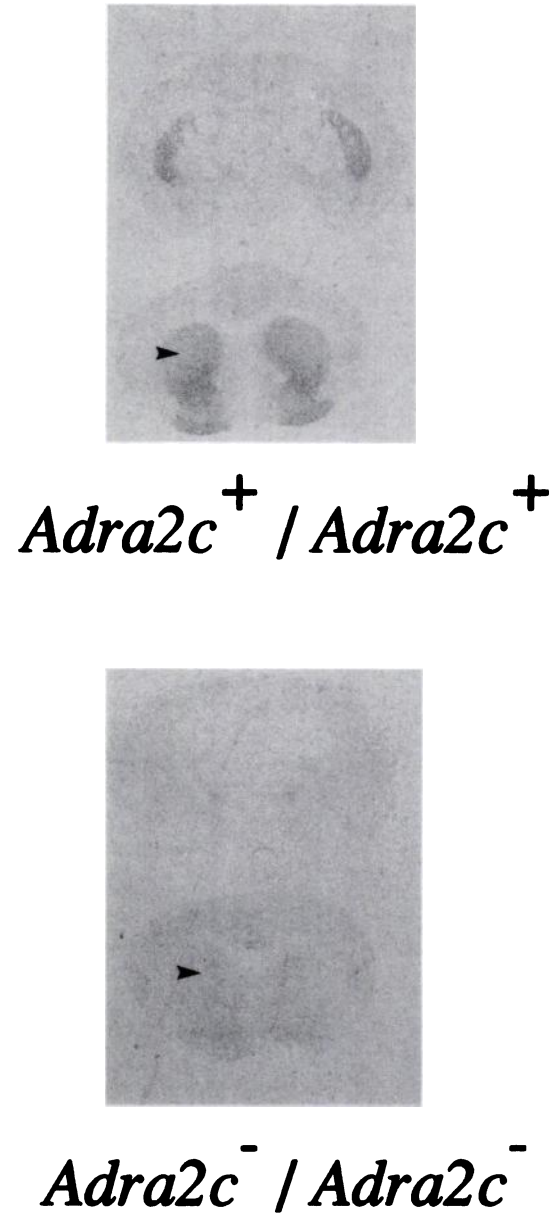

Fig. 4. Receptor autoradiography. Distribution of α_{2C} binding sites in *Adra2c*$^+$/*Adra2c*$^+$ and *Adra2c*$^-$/*Adra2c*$^-$ mouse brain sections, as detected by [^{3}H]rauwolscine autoradiography. *Top two panels*, two different planes of a section from a *Adra2c*$^+$/*Adra2c*$^+$ mouse brain; *bottom two panels*, section from a *Adra2c*$^-$/*Adra2c*$^-$ mouse brain. Samples were masked with 100 nM oxymetazoline to block radioligand binding to α_{2A}-ARs. *Arrowheads*, region of the caudate putamen.

TABLE 1

Radioligand binding to the caudate putamen in *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ mouse brain sections

[^{3}H]Rauwolscine binding in the presence of 100 nM oxymetazoline was determined for the region of the caudate putamen by autoradiography and quantitative image analysis as described in Materials and Methods. Values represent two independent experiments from one *Adra2c*$^+$/*Adra2c*$^+$ mouse, two *Adra2c*$^+$/*Adra2c*$^-$ mice, and two *Adra2c*$^-$/*Adra2c*$^-$ mice.

Genotype	Binding
	fmol/mg of tissue
Adra2c$^+$/*Adra2c*$^+$	22.0
	22.0
Adra2c$^+$/*Adra2c*$^-$	17.7
	15.9
Adra2c$^-$/*Adra2c*$^-$	7.9
	5.8

the [^{3}H]rauwolscine binding was largely concentrated in the caudate putamen. In the *Adra2c*$^-$/*Adra2c*$^-$ mice, the autoradiographic signal over the region of the caudate putamen was reduced to a level similar to that of the rest of the brain.

In the examination of these results, the limitations of the assay must be considered. At 0.8 nM, [^{3}H]rauwolscine does not saturate all of the α_{2C}-AR binding sites. Therefore, the results may underestimate maximum α_{2C}-AR density. However, nonspecific binding is determined by incubation with 100 μM epinephrine (see Materials and Methods), which may displace [^{3}H]rauwolscine from non-α_2-AR binding sites, leading to an overestimation of the amount of α_{2C}-ARs expressed (particularly in *Adra2c*$^-$/*Adra2c*$^-$ mice). Therefore, the residual binding observed in the caudate putamen probably represents not α_{2C}-AR binding but, rather, a combination of a small amount of α_{2A}-AR and non-α_2-AR [^{3}H]rauwolscine binding sites.

Striatal membrane preparations from seven animals from each group were used to determine receptor affinity (K_d) and receptor density (B_{max}) values in this brain region. Receptor affinities for [^{3}H]RX821002 were similar in membranes from *Adra2c*$^+$/*Adra2c*$^+$, *Adra2c*$^+$/*Adra2c*$^-$, and *Adra2c*$^-$/*Adra2c*$^-$ mice (0.37–0.44 nM). The binding capacity was lowest in membranes from *Adra2c*$^-$/*Adra2c*$^-$ mice (150 ± 3 fmol/mg of protein), intermediate in *Adra2c*$^+$/*Adra2c*$^-$ mice (196 ± 6 fmol/mg), and highest in *Adra2c*$^+$/*Adra2c*$^+$ mice (221 ± 10 fmol/mg). The reduced [^{3}H]RX821002 binding in *Adra2c*$^-$/*Adra2c*$^-$ mice, compared with wild-type *Adra2c*$^+$/*Adra2c*$^+$ mice (72 fmol/mg), was comparable to the density of α_{2C}-AR binding sites in striatal membranes from *Adra2c*$^+$/*Adra2c*$^+$ wild-type mice, as determined with [^{3}H]rauwolscine and 100 nM oxymetazoline to block α_{2A}-ARs (B_{max} of 83 ± 7 fmol/mg and K_d of 0.72 nM). Specific binding of [^{3}H]rauwolscine to striatal membranes of *Adra2c*$^-$/*Adra2c*$^-$ mice and *Adra2c*$^+$/*Adra2c*$^-$ mice was too low to yield reliable estimates of B_{max} and K_d.

Expression of other α_2-AR subtype mRNAs in *Adra2c*-mutant animals. The existence of mice lacking a single subtype of α_2-ARs provides an opportunity to determine whether other α_2-AR subtypes are up-regulated to compensate for the loss of *Adra2c* function. To address this question, we stripped and reprobed Northern blots with probes from the mouse *Adra2a* and *Adra2b* genes (Fig. 2). The *Adra2a*(Coding) probe detected a single 3.9-kb mRNA in brain, which did not vary with the genotype at the *Adra2c* locus. Likewise, the *Adra2b*(Coding) probe detected a 4.3-kb message in kidney that did not vary with the *Adra2c* genotype. Furthermore, there was no evidence for the up-regulation of *Adra2b* RNA levels in the brains of *Adra2c*$^-$/*Adra2c*$^-$ mice. These data are consistent with binding data from striatum and indicate that up-regulation of *Adra2a* or *Adra2b* does not compensate for the loss of *Adra2c* in *Adra2c*$^-$/*Adra2c*$^-$ mice.

Discussion

Using gene-targeting techniques, we have successfully introduced a mutation into the mouse *Adra2c* gene, which is the homolog of the human α_2-C4 and rat *RG10* α_2-AR genes. The receptor proteins encoded by *Adra2c*, α_2-C4, and *RG10* have been pharmacologically classified as α_{2C}-ARs based on their binding to a panel of subtype-selective agonists and antagonists (for review, see Ref. 45).

Based on Northern blotting and RT-PCR studies, *Adra2c*$^-$/*Adra2c*$^-$ mice do not produce a transcript capable of encoding a functional α_{2C}-AR protein. In addition, we could detect no significant up-regulation of *Adra2a* or *Adra2b* mRNA levels in the brains or kidneys of *Adra2c*$^-$/*Adra2c*$^-$ mice. These RNA studies were corroborated by receptor autoradiographic results from brain sections, where [^{3}H]rauwolscine binding (in the presence of oxymetazoline to mask α_{2A}-ARs) was markedly reduced in the caudate putamen, the brain area normally expressing the greatest proportion of α_{2C}-ARs.

A corresponding reduction in total α_2-AR ligand binding in striatal membranes from *Adra2c*$^-$/*Adra2c*$^-$ mice, as estimated by saturation analysis with the non-subtype-selective radioligand [^{3}H]RX821002, was noted. This indicates that the expression of other α_2-AR subtypes was not markedly up-regulated to compensate for loss of *Adra2c* expression in the caudate putamen. In our experiments with striatal membrane preparations, α_{2C}-type binding, determined with [^{3}H]rauwolscine and 100 nM oxymetazoline, appeared to represent approximately 38% of total α_2-AR ligand binding in this brain area. This is in line with receptor autoradiographic results from rat brain, where a low concentration of [^{3}H]rauwolscine labeled 13% of the sites labeled by the nonselective ligand [^{3}H]idazoxan in the fundus striatum, 49% in the nucleus accumbens, and 80% in the dorsal caudate putamen (40). Bearing in mind the methodological limitations of these receptor binding assays, it may be concluded that α_{2C}-ARs represent a significant proportion of total α_2-AR ligand binding in the striatum of wild-type mice, sufficient to lead to a detectable reduction in total α_2-AR ligand binding in striatal membranes from *Adra2c*$^-$/*Adra2c*$^-$ mice.

Surprisingly, *Adra2c*$^-$/*Adra2c*$^-$ animals are viable and fertile and appear grossly normal, indicating that the *Adra2c* receptor is not required for mouse embryonic development or adult reproductive function. Of the three pharmacologically defined α_2-AR subtypes (α_{2A}, α_{2B}, and α_{2C}), the least functional information is known about the α_{2C} subtype. Although it was originally isolated from an opossum kidney cell line and was believed to represent an interspecies variation of the α_{2B} subtype defined in neonatal rat lung (46), it is now clear that the α_{2C}-AR is a distinct receptor (47) encoded by a separate gene that is conserved across species (10, 12, 15, 48). In rats and mice, it is fairly straightforward to differentiate the α_{2B}- and α_{2C}-ARs from the α_{2A}-AR by *in vitro* radioligand

binding assays (12, 21, 49). It is more difficult, however, to differentiate the α_{2B} and α_{2C} subtypes from each other, because both receptors have high affinity for prazosin and low affinity for oxymetazoline.

To date, the majority of α_2-AR functions identified pharmacologically appear to be mediated by a receptor with low prazosin affinity, presumably the α_{2A} subtype. Stimulation of platelet aggregation, modulation of insulin release from pancreatic β cells (50), inhibition of lipolysis (51), and modulation of neurotransmitter release from presynaptic nerve terminals in the vas deferens and submandibular gland (52) have all been classified as α_{2A} responses. Immunohistochemical results also support the view that the presynaptic receptors of central noradrenergic neurons are of the α_{2A} subtype and that α_{2A}-ARs are involved in central cardiovascular control mechanisms (53). In contrast, prazosin has moderately high potency at the presynaptic α_2-AR in rat atrium (54) and human kidney (55) and at the postsynaptic α_2-AR in human saphenous vein (52) and rabbit vascular smooth muscle (56). It has been difficult to determine whether these receptors represent the α_{2B} and/or the α_{2C} subtypes, but these questions can now be answered by studying *Adra2c*$^{-}$/*Adra2c*$^{-}$ mice.

Adra2c^{-}/*Adra2c*$^{-}$ animals will be useful for evaluating the role of the α_{2C}-AR in the central nervous system. The greatest α_{2C}-AR expression is found in the brain, with high levels of mRNA being detected in basal ganglia, olfactory tubercle, hippocampus, and cerebral cortex (25, 28). This pattern of mRNA localization suggests that the α_{2C}-AR may play a role in higher behavioral functions such as the control of conscious movement or in learning and memory.

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