Mice Lacking D_5 Dopamine Receptors Have Increased Sympathetic Tone and Are Hypertensive

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Dopamine is an important transmitter in the CNS and PNS, critically regulating numerous neuropsychiatric and physiological functions. These actions of dopamine are mediated by five distinct receptor subtypes. Of these receptors, probably the least understood in terms of physiological functions is the D_5 receptor subtype. To better understand the role of the D_5 dopamine receptor (DAR) in normal physiology and behavior, we have now used gene-targeting technology to create mice that lack this receptor subtype. We find that the D_5 receptor-deficient mice are viable and fertile and appear to develop normally. No compensatory alterations in other dopamine receptor subtypes were observed. We find, however, that the mutant mice develop hypertension and exhibit significantly elevated blood pressure (BP) by 3 months of age. This hypertension appears to be caused by increased sympathetic tone, primarily attributable to a CNS defect. Our data further suggest that this defect involves an oxytocin-dependent sensitization of V_1 vasopressin and non-NMDA glutamatergic receptor-mediated pathways, potentially within the medulla, leading to increased sympathetic outflow. These results indicate that D_5 dopamine receptors modulate neuronal pathways regulating blood pressure responses and may provide new insights into mechanisms for some forms of essential hypertension in humans, a disease that afflicts up to 25% of the aged adult population in industrialized societies.

Key words: D_5 receptor; gene knock-out; hypertension; sympathetic tone; oxytocin; vasopressin

Dopamine is an important neurotransmitter in the brain as well as the periphery and plays a critical role in regulating numerous locomotor, neuroendocrine, cognitive, and emotional functions. Dysregulation of dopaminergic systems has also been hypothesized to underlie several neuropsychiatric and endocrine disorders, including Parkinson's disease, schizophrenia, Tourette's syndrome, and hyperprolactinemia. The actions of dopamine are mediated by five distinct receptor subtypes that belong to the G-protein-coupled receptor super-family and are divided into two major subgroups, D_1-like and D_2-like, the basis of their structure and pharmacology (Neve and Neve, 1997). The D_1-like subfamily consists of the D_1 and D_5 subtypes (also called D_{1A} and D_{1B}, respectively), both of which transduce their signals by increasing intracellular cAMP levels. The D_2-like subfamily consists of the D_2, D_3, and D_4 receptors, all of which can diminish cAMP production as well as regulate the activity of various ion channels. Although the D_1-like and D_2-like subfamilies can be differentiated pharmacologically, it is difficult to discriminate between receptors within each subfamily using selective ligands. This has led to uncertainties in ascribing specific physiological and behavioral functions to individual receptor subtypes. Investigators have approached this issue, in part, by creating genetically altered animals that lack individual receptor subtypes. Thus far, mice lacking D_1, D_2, D_3, or D_4 receptors have been produced, all of which have exhibited informative phenotypes (Sibley, 1999; Glickstein and Schmauss, 2001). This has resulted in the elucidation of receptor functions that could not have been obtained through other means such as the predominant presynaptic and postsynaptic roles of the D_{2S} and D_{2L} receptor isoforms, respectively (Usiello et al., 2000).

The D_5 dopamine receptor (DAR) has generated significant interest because of its relatively high affinity for dopamine, compared with other DARs, and its purported constitutive activity (Sunahara et al., 1991; Tiberi and Caron, 1994). This has suggested that the D_5 DAR may be activated in the absence or presence of low concentrations of endogenous agonist. Although the D_5 DAR is functionally coupled to the activation of adenylate

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cyclase, recent studies suggest that the D_5 DAR may also modulate $GABA_A$ receptor-mediated activity through both second messenger cascades (Yan and Surmeier, 1997) as well as through direct receptor–receptor interactions (Liu et al., 2000). Localization of the D_5 DAR in the brain has revealed a widespread distribution, with the highest expression in the cerebral cortex, hippocampus, and basal ganglia (Ariano et al., 1997; Ciliax et al., 2000). Interestingly, recent reports have suggested a possible association of the D_5 DAR gene with schizophrenia (Muir et al., 2001) or substance abuse (Vanyukov et al., 1998). D_5 DARs are also expressed in the hypothalamus, where they may regulate circadian rhythms (Rivkees and Lachowicz, 1997) and female sexual behaviors (Apostolakis et al., 1996a,b). Within the periphery, D_5 DARs have been found in adrenal tissue (Dahmer and Senogles, 1996), kidney (Sanada et al., 2000), and also the gastrointestinal tract, where they may exert a protective effect on the intestinal mucosa (Mezey et al., 1996). To further elucidate the physiological roles of the D_5 DAR, we have now used gene-targeting technology to generate mice lacking functional D_5 DARs.

MATERIALS AND METHODS

Construction of targeting vector and gene targeting. The targeting construct "pD5KO" contained a 9.6 kb genomic fragment of the mouse D_5 receptor gene isolated from a 129/Sv genomic library. This genomic sequence was subcloned as an *Eco*RV–*Nhe*I fragment into the gene targeting vector pPNT (Tybulewicz et al., 1991). Through a number of intermediate subcloning steps, a neomycin resistance gene was ligated, in reverse orientation, at the unique *Sfi*I site of the D_5 receptor gene, thus disrupting the reading frame within the coding region. The length of D_5 receptor genomic DNA flanking the neomycin gene in the targeting vector was 6.9 kb (3′) and 2.7 kb (5′). The targeting vector pD5KO was subsequently electroporated into the J1 line (Li et al., 1992) of embryonic stem (ES) cells, and simultaneous G418 positive selection and gancyclovir negative selection were used to enrich for ES cell colonies with successful gene targeting. Southern hybridization analysis was used to examine DNA from ES colonies for gene-targeting events. Homologous recombination at the 5′ end of the targeting construct was detected by digesting ES cell genomic DNA with *Nco*I and hybridizing the Southern blot with probe A (see Results). The normal allele was 6 kb in length, and the recombinant allele was 3.4 kb. Probe B (see Results) was hybridized to Southern blots of ES genomic DNA cut with *Kpn*I to detect homologous recombination at the 3′ end. The normal allele was 28 kb, and the length of the recombinant allele was 18 kb. A hybridization probe, derived from the Neo gene, detected a single 3.4 kb band on *Nco*I Southern blots for those ES colonies in which gene targeting was successful; bands different in length from 3.4 kb on these blots indicated random integration of one of more copies of the targeting vector into the ES cell genome.

Mutant mouse generation and genotyping. C57BL/6 blastocysts were injected with ES cells from five different recombinant ES colonies and implanted into foster mothers. The 129/Sv–C57BL/6 chimeric offspring produced by blastocyst injection were bred with C57BL/6 mice to pass the recombinant D_5 receptor allele from the germline of these chimeras to an F1 generation. Southern blots of *Nco*I- or *Kpn*I-digested genomic DNA from mouse tail biopsies, hybridized with probes A or B, respectively, were used to detect germline passage of the recombinant D_5 receptor allele in the F1 generation mice. Homozygous mutant (−/−) and wild-type (+/+) mice were generated from heterozygous mouse intermatings. In the later stages of this study, the mice were genotyped using a PCR-based method involving amplification from mouse genomic DNA isolated from tail biopsies. Oligonucleotide primers were designed to flank the *Sfi*I restriction site into which the neomycin cassette was ligated. Primer 1 (5′-ACTCTCTTAATCGTCTGGACCTTG-3′) and primer 2 (5′-TCGCAGGCTGGGGTCAGGTTCGCA-3′) were used to amplify the wild-type allele, whereas primer 3 (5′-TGATCAACTAG-TGCCCGGGCGGTA-3′), which was unique to the neomycin cassette, was used with primer 1 to amplify the recombinant allele. The PCR reaction used 0.2 μg of genomic tail DNA in a 50 μl reaction (50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM of each forward and reverse primer with 2.5 U *Taq*DNA polymerase). The initial cycle of amplification was as follows: denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 2 min. The reaction then was carried through 30 cycles consisting of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min. The extension time at 72°C for the final cycle was 10 min.

Immunohistochemistry and in situ *hybridization.* The immunohistochemical analysis of the D_5 receptor protein was performed as described previously (Ariano et al., 1997). Adult male wild-type and homozygous mutant mice were killed, the brains were removed, and 10-μM-thick fresh-frozen brain sections were generated. Tissue sections from mutants and wild-type brains were processed simultaneously. *In situ* hybridization histochemistry was performed as described (Malik et al., 1996).

Receptor autoradiography. Brains from wild-type and D_5 mutant mice ($n = 4$ each) were removed after decapitation, frozen on dry ice, and stored at −80°C. Brain sections in the coronal plane were cut at 12 μm thickness and thaw-mounted on Superfrost plus slides (Fisher Scientific, Pittsburgh, PA). One set of slides from each animal that represented forebrain and midbrain regions was used for labeling the oxytocin receptor (OTR), whereas another set was used for labeling the vasopressin-1a receptor (VP). OTR autoradiography was performed using ^{125}I–labeled ornithine vasotocin analog [D$(CH_2)_5$-[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}], ^{125}I-OTA; specific activity, 2200 Ci/mmol; NEN Life Sciences Products, Boston, MA; NEX 254] as described previously (Insel and Shapiro, 1992). VP autoradiography was performed using ^{125}I-labeled linear VP ligand [HO-phenylacetyl1-D-Tyr(Me)^2-Phe^3-Gln^4-Asn^5-Arg^6-Pro^7-Arg^8-NH_2; specific activity, 2200 Ci/mmol; NEN Life Sciences Products; NEX 310] as described previously (Young et al., 2000).

Receptor binding assays. Homogenate radioligand binding assays using striatal or kidney membrane preparations were performed as described previously for D_1-like receptors (Jiang and Sibley, 1999) and D_2-like receptors (Schetz et al., 2000). [^{3}H]-SCH-23390 (DuPont/NEN; 71.3 Ci/mmol) was used to label D_1-like receptors, whereas [^{3}H]-methylspiperone (DuPont/NEN; 84 Ci/mmol) was used to label D_2-like receptors. Adult male or female wild-type and homozygous mutant mice were killed, the brains were removed, and the corpus striatum was rapidly dissected and immediately frozen before subsequent membrane preparations and radioligand binding assays. Membrane protein concentrations were determined with the bicinchoninic acid protein reagent (Pierce, Rockford, IL) and a BSA standard curve.

Blood pressure studies. The mice were anesthetized with pentobarbital (50 mg/kg, i.p.), placed on heated board to maintain body temperature at 37°C, and tracheotomized (PE 90). Catheters were inserted into the femoral vessels and right jugular vein (PE 50 heat-stretched to 180 μm tip) for fluid administration, blood drawing, and blood pressure (BP) monitoring. After a 60 min stabilization period after the surgical procedures, the following agents were infused intravenously in random order: [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 2-(*O*-methyl)tyrosine]-Arg^8-vasopressin (Peninsula Laboratories, San Carlos, CA) at 10 μg/kg over 30 sec; BQ610 (Peninsula Laboratories) at 100 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 10 min; BQ788 (Peninsula Laboratories) at 6.6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ for 15 min; phentolamine (RBI, Natick, MA) at 5 $ng \cdot kg^{-1} \cdot min^{-1}$ for 30 min; losartan (Merck, Philadelphia, PA) at 3 mg/kg over 30 sec; GYKI 52466 (RBI) at 8 mg/kg over 30 sec; and CNQX (RBI) given at 1 mg/kg over 30 sec. The effects of these drugs, if any, on blood pressure and heart rate were monitored for 20–45 min. The blood pressure was allowed to stabilize at pre-infusion values for 30–60 min before the administration of subsequent drugs. In preliminary studies, the nonglutamatergic antagonists were shown to completely block the vasopressor effects of their respective agonists: arginine-vasopressin, phenylephrine, endothelin-1, and angiotensin II given over 30 sec at volumes of 40 μl (data not shown). In some mice, an oxytocin antagonist, D(CH2)$_5$[Tyr(Me)^2, Thr^4, Thy-NH_2]OVT (Bachem AG, Torrance, CA) was administered intraperitoneally (0.3 μg/kg) 12 and 24 hr before blood pressure determination. The blood pressures were determined before oxytocin antagonist administration under a short-acting anesthetic agent, 2,2,2-tribromoethanol. The blood pressure effects of the V_1 vasopressin receptor antagonist and GYKI 52466 were subsequently tested under pentobarbital anesthesia as described above.

In some 2,2,2-tribromoethanol-anesthetized mice, blood pressures were measured during the placement of a femoral artery catheter, coated with 5% heparin complex, that was threaded upward and out of a 5 mm incision at the nape of the neck. Analgesia (buprenorphine) was given during the recovery period and continued on the first day after surgery. One-third ml of a sterile solution (1/2 mg plasmin and 1000 U heparin/ml of sterile saline) was used to flush the catheter immediately and every 2 d

thereafter. Blood pressures were subsequently measured in freely moving, unanesthetized mice, 1–3 d after catheter placement.

Determination of catecholamine levels. The adrenal glands were homogenized with 0.1 M $HClO_4$ and centrifuged at 6000 × *g* for 20 min at 4°C, and catecholamine concentrations were determined by HPLC and electrochemical detection (Caramona and Soares-da-Silva, 1985).

RESULTS

Generation of D_5 DAR-deficient mice

A PCR-generated cDNA encoding the rat D_5 DAR was used to screen a 129/Sv mouse genomic library to isolate the mouse D_5 DAR gene. Several clones were isolated and characterized through partial sequencing and restriction mapping to confirm that they encoded the D_5 DAR gene. A restriction map of the mouse D_5 DAR gene is shown at the top of Figure 1*A*. To inactivate the D_5 DAR gene, a neomycin resistance gene was ligated, in reverse orientation, into a unique *Sfi*I site of the D_5 receptor gene, thereby disrupting the reading frame within the coding region (Fig. 1*A*). A stop codon was engineered into the proximal neomycin gene linker such that the recombinant D_5 receptor would be prematurely truncated subsequent to Gly-190 in the second extracellular loop of the receptor. A total of 216 transfected ES cell lines were screened by Southern blotting (Fig. 1*B*), resulting in the identification of six cell lines exhibiting homologous recombination. ES cells amplified from five of these lines were used to generate chimeric male mice that were subsequently bred with C57/BL6 females. Only one line (Fig. 1*B*, *112*) produced chimeras capable of transmitting the mutant allele to their offspring. Southern analysis of tail DNA from the progeny of heterozygous matings revealed the predicted restriction patterns for wild-type (+/+), heterozygote (+/−), and mutant (−/−) genotypes (Fig. 1*C*).

The D_5 DAR null mutant mice develop normally with no notable differences from wild-type littermates with respect to appearance, body weight, or home cage behaviors. The mutant allele appears to be inherited in a Mendelian manner, and the null mutants were fertile and capable of reproduction. Histological examinations revealed no abnormalities in major organ systems, and no obvious neurological or behavioral anomalies were noted. A more detailed behavioral characterization of these animals is presented elsewhere (Holmes et al., 2001).

Confirmation of D_5 DAR gene disruption

To confirm the disruption of the D_5 DAR gene, we took two different approaches. Because the D_1 and D_5 DARs are pharmacologically similar, there are no radioligands that selectively label the D_5 subtype, and because as the D_1 DAR is more prevalent than the D_5, binding assays with D_1-like ligands will predominantly label the D_1 DAR with little signal contributed by the D_5 subtype. Thus, we initially verified that the recombinant transcript was expressed by the mutant animals by performing RT-PCR analysis using RNA extracted from mouse brains. Figure 2*A* shows our strategy for the identification and detection of the wild-type and mutant transcripts by PCR. We designed three primers: 1 and 2 are unique to the coding sequence of the D_5 receptor and flank the *Sfi*I site into which the neomycin resistance gene was inserted, whereas primer 3 is unique to the inserted *neo* gene sequence. Amplification with primers 1 and 2 should only result in a 702 bp fragment from the wild-type allele, whereas amplification with primers 1 and 3 should only result in a 468 bp fragment from the mutant allele. Although theoretically primers 1 and 2 could also give rise to a very large fragment from the

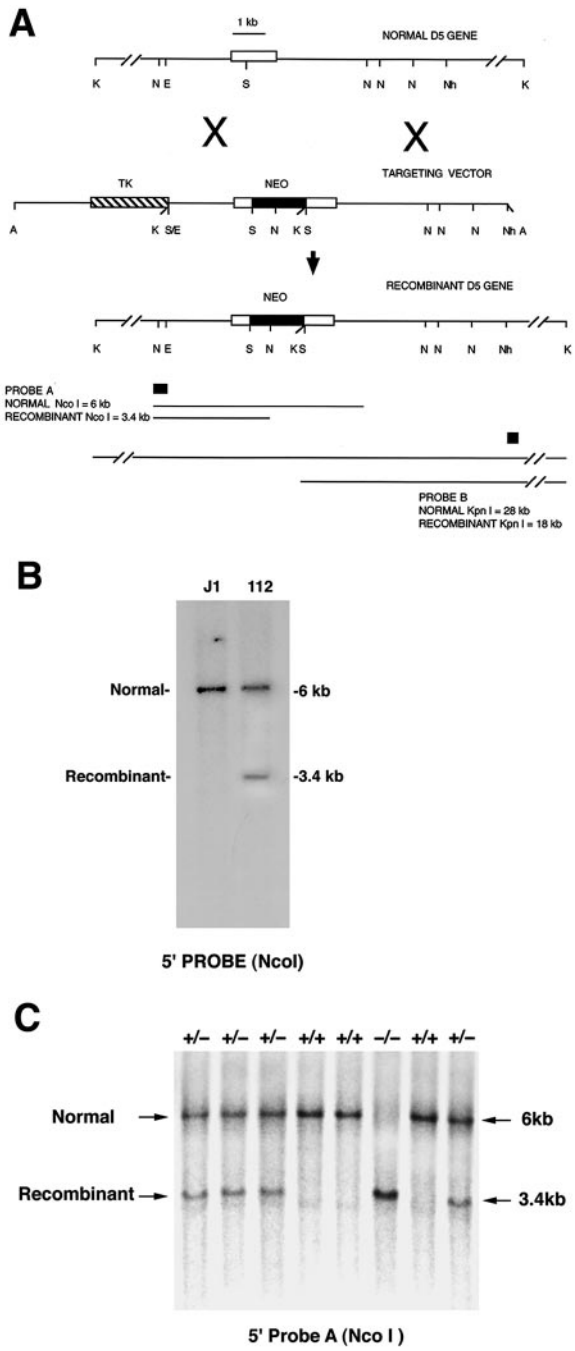

Figure 1. Generation of D_5 receptor–deficient mice. *A*, The D_5 receptor gene, targeting construct, and mutant loci of the D_5 gene. The *open box* represents the coding region of the D_5 gene, the *solid box* depicts the neomycin phosphotransferase gene, and the *stippled box* represents the herpes simplex thymidine kinase gene. Probes used in Southern hybridization analysis and predicted lengths of restriction fragments are also shown. *A*, AscI; *E*, *Eco*RV; *K*, *Kpn*I; *N*, *Nco*I; *Nh*, *Nhe*I; *S*, *Sfi*I, *S*/*E*, ligation junction of SrfI and *Eco*RV sites. *B*, Genomic analysis of targeted ES cell DNA. Recombination was detected at the 5′ end by digesting ES cell DNA with *Nco*I and hybridizing the Southern blot with probe A. *J1* represents the wild-type ES cell DNA, and *112* represents a targeted ES cell line exhibiting homologous recombination. *C*, Representative pedigree obtained from genotyping a litter of mice derived from crossing heterozygote parents. The genomic DNA was obtained from tail biopsies and digested with *Nco*I. The blot was subsequently hybridized with *Probe A* as described in *B*.

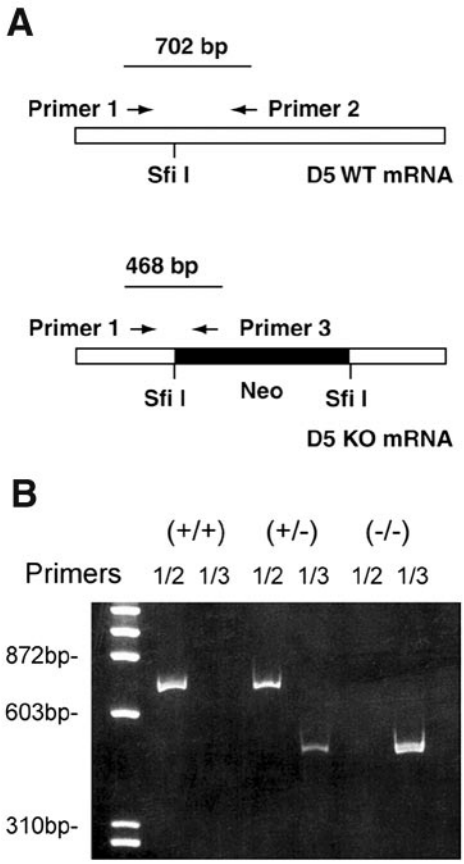

Figure 2. Documentation that the D_5 mutant allele is expressed *in vivo*. *A*, RT-PCR strategy for detection of the wild-type and mutant D_5 receptor alleles in mouse tissues. *Top*, The coding region of the wild-type allele is shown with the unique *Sfi*I restriction site. *Primers 1* and *2* are derived from the coding region sequence. The predicted wild-type fragment is 702 bp in length. *Bottom*, The recombinant gene is shown with the neomycin resistance gene insert. *Primer 3* is derived from the neomycin resistance gene. The predicted mutant fragment is 468 bp in length. *B*, RT-PCR analysis of RNA isolated from brain tissue. Wild-type (+/+), heterozygous (−/+), and mutant (−/−) animals, identified by genotyping, were killed, brains were removed, and total RNA was isolated using the RNEasy Mini Kit (Qiagen, Hilden, Germany) and used for first-strand cDNA synthesis (Superscript II, Invitrogen, Gaithersburg, MD). An aliquot of this cDNA was subjected to amplification by PCR using the genotyping procedure described in Materials and Methods. The primers used in each amplification reaction are indicated at the *top* of each *lane*; the size of the fragments is indicated on the *left*.

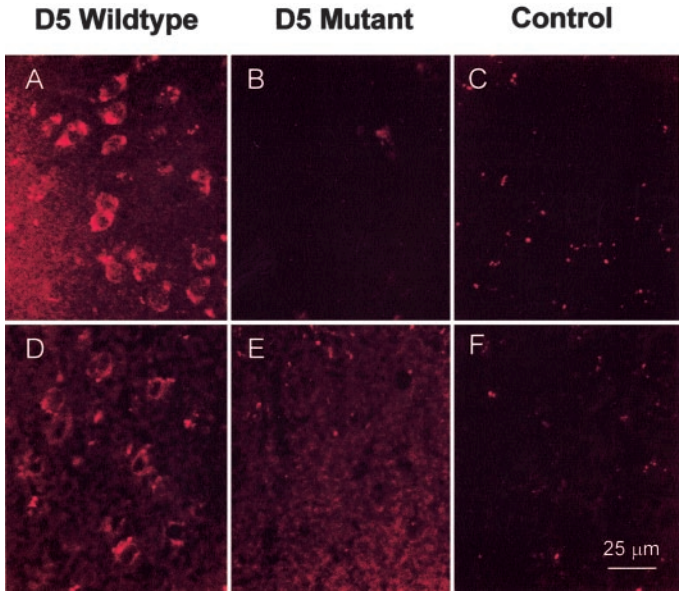

Figure 3. Immunohistochemical detection of D_5 receptor protein in wild-type and mutant mouse brains. Fresh-frozen mouse brains were mounted in the coronal plane and sectioned at 10 μm. Sections derived from frontal cortex were subsequently processed as described in Materials and Methods. *A–C* show results with antisera P14, which is directed to an extracellular epitope in the third external loop of the receptor. *D–F* show results with antisera C14, which is directed to an intracellular epitope in the third cytoplasmic loop of the receptor. *C* and *F* show wild-type tissue processed in the absence of primary antisera. Scale bar, 25 μm.

mutant allele, the PCR conditions were not optimized for this to occur. Figure 2*B* shows an RT-PCR experiment using RNA extracted from the brains of homozygous wild-type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice that had been genotyped via Southern blotting as shown in Figure 1*C*. As can be seen, all of the genotypes gave the predicted pattern of PCR fragments. Similar RT-PCR data were generated using RNA extracted from kidneys of wild-type and mutant genotypes (data not shown). These results indicate that the mutant mice are expressing the recombinant transcript as expected.

Disruption of the D_5 DAR gene was further confirmed by directly examining the expression of the D_5 receptor protein using immunohistochemistry. We have previously described selective antisera for labeling the D_5 receptor protein in rat brain (Ariano et al., 1997). Two antisera were generated using peptides derived from the third extracellular and third intracellular loops of the D_5 DAR. Both of these epitopes are "downstream" of the truncation site in the recombinant D_5 DAR and should not be expressed in the mutant animals. Figure 3 shows immunofluorescence in fresh-frozen sections of frontal cortices from wild-type and mutant mouse brains. Both anti-D_5 DAR antisera detected neurons in the frontal cortices of wild-type animals (Fig. 3*A*,*D*), analogous to results in rat brain tissue (Ariano et al., 1997). In contrast, no staining was observed in brains from the D_5 mutant animals (Fig. 3*B*,*E*), and the fluorescence was at background levels (*C*, *F*). Taken together, the data in Figures 2 and 3 confirm that the D_5 DAR gene was disrupted in the mutant animals.

D_1- and D_2-like receptor binding is normal in the D_5 mutant mice

As part of our initial characterization of the D_5 mutant mice, we wished to assess whether there were any alterations in the expression of other dopamine receptor subtypes, perhaps arising as a consequence of developmental compensation. We thus performed radioligand binding assays using striatal membrane homogenates because the striatum is one of the regions of highest expression for the D_1 and D_2 receptor subtypes and also contains D_3 and D_4 receptors (Ariano, 1996). Figure 4*A* shows a saturation radioligand binding experiment using the D_1-like selective antagonist [^{3}H]SCH-23390 in membranes prepared from wild-type and mutant mice. As can be seen, there were no differences between the genotypes. Similar results were obtained via D_1 receptor radioligand binding assays in kidney membranes derived from both genotypes (data not shown). Because only the D_1 receptor is being labeled in the D_5 mutant mice, these results indicated that there were no compensatory alterations in this receptor subtype and further illustrated the predominance of the D_1 DAR. In other studies, we also used D_1-selective antisera and

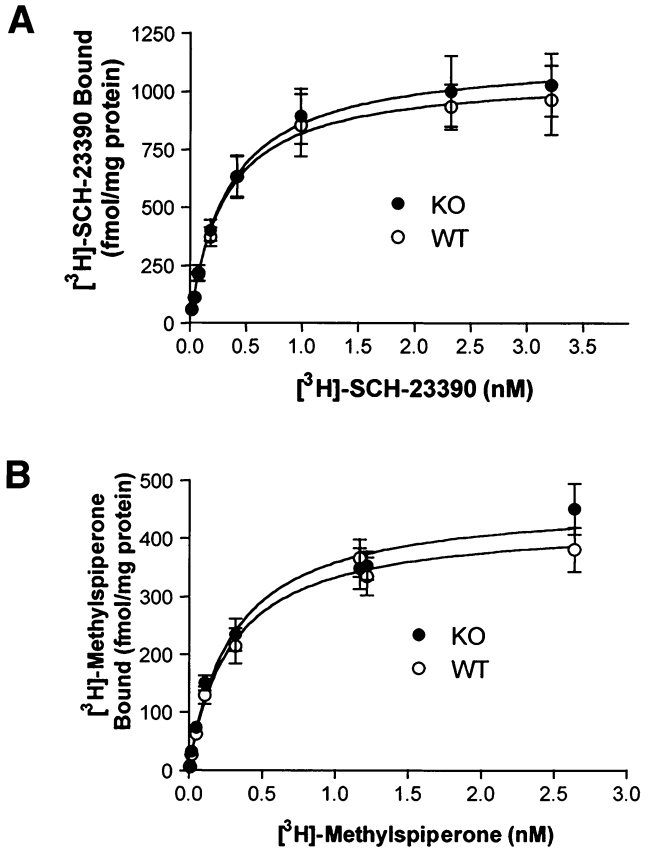

Figure 4. D_1- and D_2-like receptor binding assays in striatal membrane homogenates of D_5 mutant mice. Striatal membranes were prepared from wild-type (*WT*) and mutant (*KO*) mice, and radioligand binding assays were performed as described in Materials and Methods with the indicated concentrations of ligands. Only specific binding is shown. The data represent the mean ± SEM values from three separate experiments using individual mice. *A*, Saturation binding analyses in striatal membranes using the D_1-like selective antagonist [^{3}H]SCH 23390. Computer analysis of the radioligand binding data resulted in the following parameters: wild-type, $K_D = 0.34 \pm 0.01$ nM, $B_{max} = 1.01 \pm 0.18$ pmol/mg protein; mutant, $K_D = 0.38 \pm 0.03$ nM, $B_{max} = 1.17 \pm 0.28$ pmol/mg protein. Using Student's *t* test, there was no significant difference between the B_{max} values ($p = 0.51$). *B*, Saturation binding analyses in striatal membranes using the D_2-like selective antagonist [^{3}H]methylspiperone. Computer analysis of the radioligand binding data resulted in the following parameters: wild-type, $K_D = 0.30 \pm 0.04$ nM, $B_{max} = 430 \pm 81$ fmol/mg protein; mutant, $K_D = 0.28 \pm 0.02$ nM, $B_{max} = 460 \pm 60$ fmol/mg protein. Using Student's *t* test, there was no significant difference between the B_{max} values ($p = 0.67$).

immunohistochemical techniques to verify that the cellular staining of the D_1 receptor was unaltered in various brain regions of the D_5 DAR-deficient mice (data not shown).

Figure 4*B* shows a saturation radioligand binding assay using the D_2-like selective antagonist [^{3}H]methylspiperone. This ligand will label the D_2, D_3, and D_4 receptors with approximately equal affinity. Within the striatum, however, ~90–95% of the D_2-like receptors are composed of the D_2 subtype, with the rest consisting of the lower abundant D_3 and D_4 receptors. As can be seen, there are no significant differences in [^{3}H]methylspiperone binding between the two mouse genotypes. Although the D_3 and D_4 receptors remain to be analyzed in greater detail, these results suggested that there were no alterations in D_2 receptor expression in the D_5 mutant animals.

The D_5 receptor mutant mice are hypertensive and have increased sympathetic tone

Several physiological parameters were examined in adult D_5 mutant mice (−/−), and comparisons were made with wild-type littermates (+/+) as well as with the parental C57BL/6 and 129/Sv mouse strains (Table 1). Although there were no differences in body or kidney weights between the D_5 +/+ and D_5 −/− genotypes, the D_5 mutant mice exhibited significantly elevated heart weights as well as elevated systolic, diastolic, and mean blood pressures. Blood pressures were also significantly elevated in the mutant animals even in the absence of anesthesia: wild-type ($n = 4$) systolic BP = 119 ± 4, mean BP = 103 ± 3, diastolic BP = 93 ± 4; mutant ($n = 5$) systolic BP = 154 ± 6, mean BP = 127 ± 8, diastolic BP = 115 ± 8; $p < 0.05$ for all wild-type versus mutant values; Student's *t* test. No gender differences were noted.

Because the 129 parental strain also showed significantly higher heart weights (Table 1), the elevated heart weights in the D_5 −/− mice could be attributable to 129-linked genes. Alternatively, the increased heart size in the mutants may represent a compensatory response to the elevation in blood pressure. To address this issue, we evaluated the cardiovascular parameters in young mice not older than 2 months of age. Table 2 shows these results. No differences were observed in the genotypes for the parameters obtained using the young mice. The blood pressures were slightly but not significantly elevated at this age, and there were no differences in the heart weights. These results indicate that the hypertension exhibited by the D_5 −/− mice was age dependent and suggest that the cardiac hypertrophy observed in the adult mutants was a result of the elevated blood pressure.

Because D_5 receptors inhibit catecholamine release from adrenal chromaffin cells (Dahmer and Senogles, 1996), we tested whether elevated adrenal catecholamines might contribute to the increased blood pressure in the null mutants. Adrenal norepinephrine and epinephrine levels are presented in Table 3. No significant differences in the absolute levels of norepinephrine or epinephrine were found between genotypes, but there was a significant elevation in the epinephrine/norepinephrine ratio in the mutant animals. Furthermore, acute adrenalectomy resulted in a greater reduction in mean arterial pressure in the D_5 mutant animals (110 ± 8 to 62 ± 6 mmHg; $n = 7$) compared with wild-type mice (88 ± 0.4 to 56 ± 5 mmHg; $n = 6$). Given these results, we wondered whether sympathetic blockade would normalize the blood pressure in the mutant mice relative to wild-type animals. Infusion of the α-adrenergic antagonist phentolamine resulted in a greater and more rapid reduction of blood pressure in the mutant mice compared with wild-type animals (Fig. 5*A*). Taken together, these experiments suggested that activation of the sympathetic nervous system occurred after disruption of the D_5 receptor gene.

Because of the low-level expression of D_5 receptors in the adrenal medulla, we thought that the increased sympathetic tone in the D_5-deficient mice might be explained more readily by a defect within the CNS. Because central sympathetic nerve responses originating in the pons and medulla are regulated by non-NMDA glutamatergic pathways (Butcher and Cechetto, 1998), we evaluated the effect of glutamatergic blockade on the blood pressure responses. Figure 5*B* shows the results of infusing the AMPA/kainate glutamatergic receptor antagonists CNQX and GYKI 52466 (Yoshiyama et al., 1995) into wild-type and mutant mice. CNQX does not cross the blood–brain barrier and had no effect on the mean arterial blood pressure in either

Table 1. Physiological measurements in adult D_5 mutant (−/−), wild-type littermates (+/+), and parental strains of mice (3–9 months old)

Variables	D_5 +/+ (n = 67)	D_5 −/− (n = 70)	C57BL/6 (n = 5)	129/Sv (n = 4)
Body weight (BW) (gm)	35 ± 1	35 ± 2	27 ± 1	30 ± 4
Heart weight (% BW)	0.40 ± 0.01	0.45 ± 0.01*	0.44 ± 0.04	0.59 ± 0.04**
Kidney weight (% BW)	1.17 ± 0.04	1.30 ± 0.08	1.30 ± 0.07	1.07 ± 0.13
Systolic blood pressure (mmHg)	103 ± 1	136 ± 4*	100 ± 1	103 ± 2
Diastolic blood pressure (mmHg)	77 ± 1	103 ± 4*	74 ± 2	80 ± 1
Mean blood pressure (mmHg)	85 ± 1	114 ± 4*	83 ± 1	87 ± 1

All values are means ± SEM. *$p < 0.05$ versus D_5 +/+; Student's t test. **$p < 0.05$ versus others; one-way ANOVA; Duncan's test.

Table 2. Physiological measurements in young (2 months old) D_5 mutant (−/−) and wild-type (+/+) mice

Variables	D_5 +/+ (n = 3)	D_5 −/− (n = 3)
Body weight (BW) (gm)	23 ± 1	21 ± 1
Heart weight (% BW)	0.48 ± 0.01	0.49 ± 0.02
Systolic blood pressure (mmHg)	99 ± 3	114 ± 7
Diastolic blood pressure (mmHg)	67 ± 2	84 ± 7
Mean blood pressure (mmHg)	78 ± 2	94 ± 7

All values are means ± SEM.

Table 3. Catecholamine levels in adrenal glands from D_5 mutant (−/−) and wild-type (+/+) mice

	D_5 +/+	D_5 −/−	p
Norepinephrine	2646 ± 200	2181 ± 314	0.25
Epinephrine	5894 ± 672	8944 ± 1819	0.15
E/NE	2.3 ± 0.4	4.1 ± 0.5	0.02

All values are means ± SEM (nanograms per gland) from five glands per genotype. p values are derived from Student's t test. *E/NE*, Epinephrine/norepinephrine.

wild-type or mutant mice. In contrast, GYK1 52466, which does cross the blood–brain barrier, reduced blood pressure in the mutant mice but had no effect on the wild-type animals. These results suggested that central non-NMDA glutamatergic pathways were abnormally activated in the null mutants, resulting in an elevation in blood pressure.

To further explore the central mechanisms underlying the hypertension in the D_5 DAR mutant mice, we investigated the role of the neuropeptide vasopressin. Arginine vasopressin (AVP) and V_1 vasopressin receptors regulate cardiovascular function and blood pressure and may be involved in human essential hypertension (Bakris et al., 1997). AVP also increases arterial blood pressure via activation of V_1 receptors in the area postrema, which projects to the nucleus tractus solitarius in the dorsomedial medulla (Migita et al., 1998). Interestingly, both NMDA and non-NMDA glutamate receptors are known to regulate the synaptic pathway between the area postrema and nucleus tractus solitarius (Aylwin et al., 1998; Migita et al., 1998). Figure 5*C* shows that infusion of the centrally acting V_1 receptor antagonist, [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 2-(*O*-methyl)tyrosine]-Arg^8-vasopressin (Bealer and Abell, 1995) reduced the mean arterial pressure in the mutant mice to normal levels yet did not significantly affect blood pressure in the wild-type animals. These results suggested that increased central V_1 receptor activity contributed to the elevated blood pressure in the D_5 mutant mice. Importantly, co-administration of the V_1 receptor antagonist and the glutamate antagonist GYKI 52466 to the mutant mice did not result in an additive reduction of blood pressure (data not shown), suggesting a common mechanism or pathway of action.

Because endothelins and angiotensin II are pressor agents that have been implicated in the pathogenesis of hypertension, we examined the cardiovascular effects of antagonizing these receptor systems (Fig. 5*D*). The endothelin A and B receptor antagonists, BQ610 (Beyer et al., 1999) and BQ788 (Allcock et al., 1995), respectively, did not affect the mean arterial blood pressure in either genotype. In contrast, the angiotensin II AT_1 receptor antagonist losartan (Asico et al., 1998) reduced the mean arterial pressure to the same extent in both genotypes. These results indicated that deletion of the D_5 receptor did not alter these peptide/receptor systems and demonstrated that the mutant mice were not selectively sensitive to depressor agents in general.

Because pretreatment of rats with the peptide oxytocin sensitizes the V_1 receptor-mediated pressor response to vasopressin (Poulin et al., 1994), we evaluated the role of oxytocin pathways in the elevated blood pressure in the mutant mice. Figure 6 shows the results from an experiment in which a centrally acting oxytocin receptor antagonist, $D(CH2)_5[Tyr(Me)^2, Thr^4, Thy\text{-}NH_2]OVT$ (Boccia et al., 1998), was administered 12 and 24 hr before the blood pressure measurements. Interestingly, pretreatment with the oxytocin antagonist alone was sufficient to reduce the systolic blood pressure to normal levels in the mutant mice, whereas there was no effect on the wild-type animals. Moreover, pretreatment with the oxytocin receptor antagonist negated any further effect on blood pressure by subsequent infusion of the glutamatergic antagonist GYKI 52466 or the V_1 receptor antagonist. These results suggested that oxytocin elevated blood pressure in the D_5 DAR-deficient mice by increasing the activity of central glutamatergic and vasopressin pathways potentially involved in regulating sympathetic outflow.

Because dopamine, acting partially via D_1-like DARs, stimulates the synthesis of vasopressin and oxytocin in the brain and pituitary (Cornish and van den Buuse, 1995; Mathiasen et al., 1996; Galfi et al., 2001), we examined their corresponding mRNA levels in the D_5 DAR-deficient mice. *In situ* hybridization histochemical analysis of vasopressin and oxytocin mRNA in the hypothalami of wild-type and mutant mice showed abundant expression of both mRNAs in the paraventricular and supraoptic nuclei (Fig. 7). Surprisingly, there was a significant decrease in the expression of vasopressin mRNA in D_5 DAR-deficient mice but no alteration in the mRNA levels for oxytocin. We also observed decreased plasma levels of AVP in the mutant mice, although this did not achieve statistical significance (wild type: 0.070 ± 0.023 ng/ml, n = 12; mutant: 0.027 ± 0.007 ng/ml, n = 12; p = 0.15).

Given the results in Figure 7, we thought it would be of interest to examine the expression of oxytocin and vasopressin V_1 receptors in the D_5 mutant brains. Receptor autoradiography was

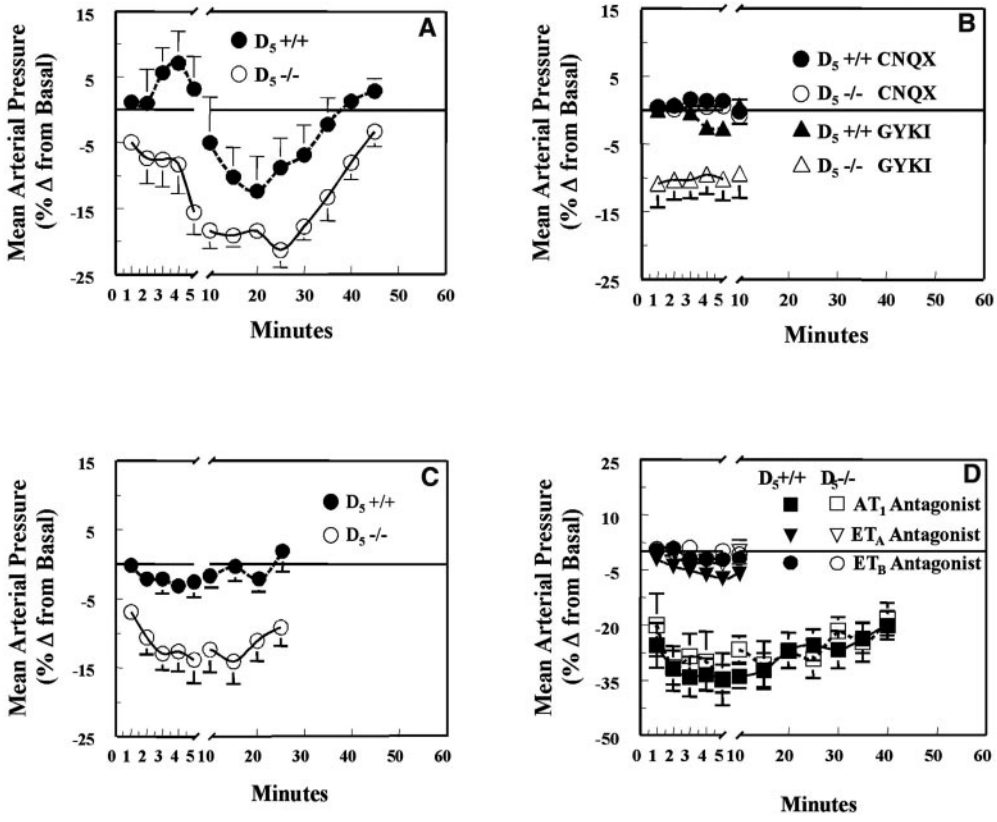

Figure 5. Mean arterial blood pressure responses in mice resulting from infusion of various pharmacological agents. Infusions were performed as described in Materials and Methods. The data represent the means ± SEM. In some cases, the error bars are smaller than the data points. *A*, The α-adrenergic antagonist phentolamine was infused into wild-type ($n = 7$) and mutant ($n = 14$). *B*, The peripherally restricted (*CNQX*) and centrally acting (*GYKI* 52466) glutamatergic antagonists were infused into wild-type and mutant mice: CNQX: wild-type ($n = 6$), mutant ($n = 6$); GYKI 52466: wild-type ($n = 9$), mutant ($n = 9$). *C*, The V_1 vasopressin antagonist, [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 2-(*O*-methyl)tyrosine]-Arg^8-vasopressin was infused into wild-type ($n = 8$) and mutant ($n = 14$) mice. *D*, The angiotensin II AT_1 receptor antagonist, losartan, and the endothelin A and B receptor antagonists, BQ610 and BQ788, respectively, were infused into wild-type and mutant mice ($n = 6$–14 per group).

performed throughout multiple serial brain sections using radioiodinated ligands for both V_1 vasopressin and oxytocin receptors. Figure 8 shows representative coronal sections through the hypothalami of wild-type and mutant mice. No consistent differences were noted between the genotypes at any level examined. Deletion of the D_5 receptor therefore did not appear to affect the expression levels of these receptors.

DISCUSSION

Since its initial discovery and characterization, the exact physiological and behavioral roles of the D_5 receptor have been difficult to clarify with certainty. This has been attributable, in large part, to the fact that the D_1 and D_5 DARs are pharmacologically indistinguishable. There are few, if any, ligands that exhibit >10-fold selectivity for either subtype (Neve and Neve, 1997). Interestingly, dopamine is one of the most selective agents demonstrating ~10-fold higher affinity at the D_5 DAR compared with the D_1. This lack of selective ligands has made it virtually impossible to selectively activate or block D_1 or D_5 receptors *in vivo*. Genetic approaches to this problem have been used by investigators using antisense technologies to downregulate D_1 or D_5 DAR expression as well as the creation of D_1 DAR-deficient mice (Sibley, 1999; Glickstein and Schmauss, 2001). These studies have demonstrated a predominant role of the D_1 receptor in regulating various locomotor, cognitive, and other behaviors. Reports of antisense "knock-down" of D_5 receptor expression have been sparse but have suggested a role for the D_5 DAR in regulating female sexual behaviors (Apostolakis et al., 1996a,b) and locomotor responses to dopaminergic agonists (Dziewczapolski et al., 1998).

In the present study, we have used gene targeting technology to generate mice completely lacking functional D_5 DARs. Documenting the inactivation of the D_5 DAR gene presented a challenge because there are no radioligands that can be used to selectively label the D_5 subtype without simultaneously labeling the D_1 DAR, and because as the D_1 receptor is more abundant relative to the D_5, radioligand binding assays with D_1-like ligands will predominantly label the D_1 DAR with very little signal being contributed by the D_5 subtype. This has been well demonstrated in the recent publication of Montague et al. (2001), who performed radioligand binding assays in mice lacking the D_1 receptor subtype. Using [^{3}H]SCH-23390, which labels both D_1 and D_5 receptors, almost all D_1-like binding was found to be ablated in the brains of these animals. There were, however, demonstrable levels of [^{3}H]SCH-23390 binding in the hippocampi of the D_1 knock-out mice. This was presumed to represent binding to the D_5 receptor because the hippocampus is a brain region of (relatively) high expression for this subtype. Although the use of radioiodinated ligands could probably detect more D_5 receptor binding sites in the D_1 DAR-deficient mice, it is clear that the D_1 receptor is more abundant than the D_5, and approaches other than radioligand binding are needed to demonstrate the absence of a functional D_5 receptor. We thus used two complementary approaches. First, we used RT-PCR analyses to show that the D_5 mutant mice were expressing the recombinant transcript as expected. Second, we used selective antisera to the D_5 DAR and showed that the D_5-deficient mice lacked specific immunohistochemical staining that was observed in wild-type mice. Taken together, these results indicate that the D_5 DAR gene was inactivated as planned.

The D_5 DAR-deficient mice were viable, appeared to develop normally, and were fertile and capable of reproduction. This latter observation was especially interesting given the antisense

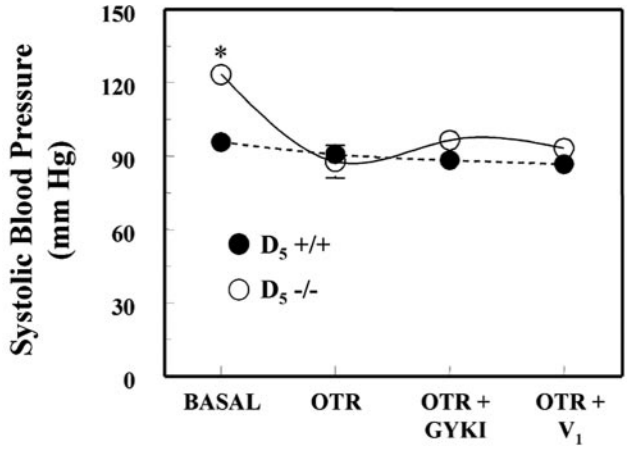

Figure 6. Blood pressure responses in mice pretreated with a centrally acting oxytocin receptor antagonist. The blood pressures were initially determined (*BASAL*) followed by two injections of the oxytocin receptor antagonist D(CH2)$_5$[Tyr(Me)^2, Thr^4, Thy-NH$_2$]OVT, as described in Materials and Methods. Systolic blood pressures were then evaluated alone (*OTR*) or subsequent to the infusion of GYKI 52466 (*OTR + GYKI*) or [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 2-(*O*-methyl)tyrosine]-Arg^8-vasopressin (*OTR + V_1*) as described in Figure 5, *B* and *C*. The data represent the mean ± SEM values from five to nine animals per group. In some cases, the error bars are smaller than the data points. *$p < 0.05$ versus other groups; ANOVA; Newman–Keuls test. Similar results were observed for diastolic blood pressures (data not shown).

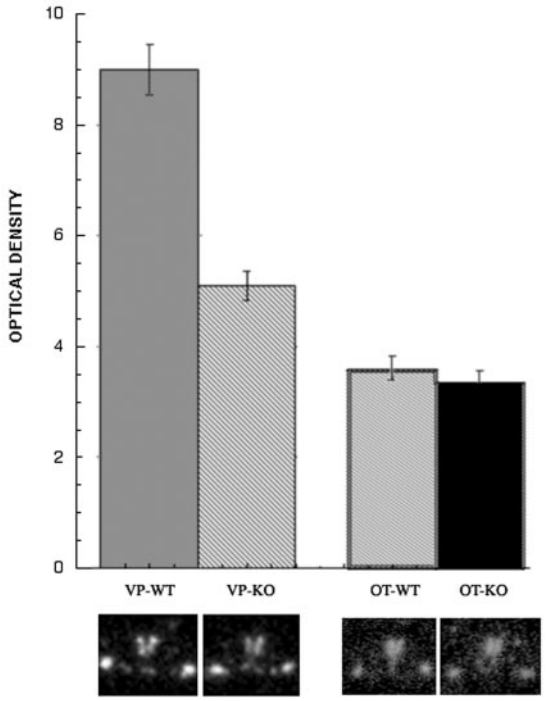

Figure 7. *In situ* hybridization histochemical (ISHH) analyses of vasopressin and oxytocin mRNA in the hypothalami of wild-type ($n = 8$) and mutant mice ($n = 9$). ISHH was performed as described in Materials and Methods, with representative images shown at the *bottom* of the figure. Phosphorimage analysis was performed by choosing a level that went through the middle of the paraventricular and supraoptic nuclei in each brain. The *y*-axis represents arbitrary optical density (OD) values assigned by the Image Gauge program of the Fuji phosphor imager. The vasopressin mRNA levels were significantly decreased in the mutant brains: OD mean ± SEM values: wild-type, 9.0 ± 0.73; mutant, 5.1 ± 0.73; $p < 0.05$; Student's *t* test.

studies (Apostolakis et al., 1996a,b) that described suppression of lordosis behavior in receptive females after D_5 DAR knock-down in the ventromedial nucleus of the hypothalamus. Despite their ability to reproduce, it will be interesting to determine whether the sexual behaviors of these animals are abnormal in any way. In general, the home cage behaviors of the D_5 DAR-deficient mice appeared normal. Casual observation could not distinguish between mutant and wild-type animals. As is reported elsewhere, however, the D_5 mutant mice did show some altered behavioral responses to dopaminergic agonist stimulation (Holmes et al., 2001). Additional experimentation using the D_5 DAR-deficient mice is currently underway to elucidate additional behavioral roles of this receptor subtype.

Because functional deletion of the D_5 DAR might result in compensatory upregulation of other dopamine receptor subtypes, especially the D_1 DAR, we examined the expression of D_1-like and D_2-like receptors in the striatum, a brain region that expresses all DAR subtypes (Ariano, 1996). Using radioligands, which label either D_1-like or D_2-like receptors, we found no differences in the receptor binding activities when comparing mutant and wild-type animals. Similarly, no alterations in D_1 receptor expression were noted using immunohistochemical methods. These results indicate that there are no compensatory alterations in the expression of the D_1 DAR and suggest that the D_2-like receptors (D_2, D_3, and D_4) are similarly unaffected.

As part of our initial characterization of the D_5 mutant animals, we discovered that they were hypertensive, exhibiting significantly elevated blood pressures. The elevation in the epinephrine/norepinephrine ratio and the greater reduction in mean arterial pressure after adrenalectomy, or with α-adrenergic blockade, in the mutant mice compared with wild-types, suggested that the hypertension was caused by increased sympathetic activity. However, because the percentage decrease in systolic blood pressure after adrenalectomy was similar in both mutant and wild-type mice, we sought to determine whether there were CNS mechanisms that may have contributed to the increase in blood pressure in the D_5 mutant mice.

Dopamine receptors, including the D_5 DAR, are present in the prefrontal cortex (Ariano et al., 1997; Ciliax et al., 2000), which projects to several brain areas involved with cardiovascular regulation (Verbene and Owens, 1998). Sympathetic responses from the prefrontal cortex are mediated within the lateral hypothalamic area (LHA) and ventrolateral medulla (VLM). Moreover, sympathetic responses originating in the prefrontal cortex and LHA are mediated by non-NMDA glutamate receptors in the VLM (Butcher and Cechetto, 1998). Indeed, CNS stimulation of non-NMDA glutamate receptors, specifically in the VLM, increases blood pressure (Chen et al., 1994; Araujo et al., 1999). Our studies suggest that the increased blood pressure in the D_5 DAR-deficient mice may be caused by activation of a sympathetic/non-NMDA glutamatergic axis because only a centrally acting non-NMDA glutamatergic antagonist decreased blood pressure in D_5 mutant mice.

The D_5 receptor may also negatively interact with oxytocin and vasopressin pathways in the prefrontal cortex and other brain areas associated with autonomic control (Ariano et al., 1997; Hermes et al., 1998; Buijs and Van Eden, 2000; Ciliax et al., 2000). Thus, V_1 vasopressin (Bealer and Abell, 1995) and oxytocin (Boccia et al., 1998) antagonists that cross the blood–brain

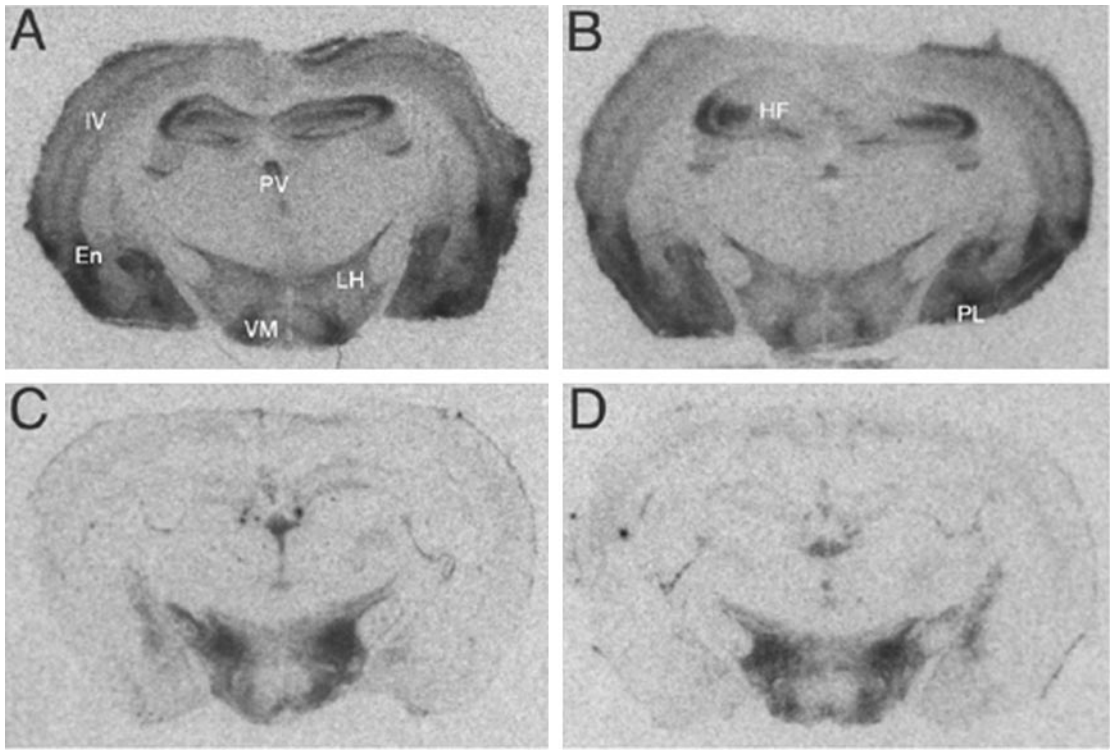

Figure 8. Receptor autoradiography of V_1 vasopressin and oxytocin receptors in brain sections of wild-type ($n = 3$) and mutant mice ($n = 3$). Representative autoradiograms of sections from wild-type (*A*, *C*) and mutant (*B*, *D*) mice through the hypothalamus show binding of the oxytocin (*A*, *B*) and vasopressin (*C*, *D*) receptor ligands. No consistent differences were noted between the wild-type and knock-out mice at any level examined. *IV*, Cortical layer 4; *En*, endopiriform nucleus; *HF*, hippocampal formation; *LH*, lateral hypothalamus; *PL*, posterolateral cortical amygdaloid nucleus; *PV*, thalamic paraventricular nucleus; *VM*, ventromedial hypothalamic nucleus. Three sets of wild-type and D_5 mutant brains were examined with similar results.

barrier were found to decrease the blood pressure in the D_5 mutant but not wild-type mice. Interestingly, the hypotensive effect of the oxytocin antagonist occurred only 24 hr after its administration and negated any further reduction in blood pressure by vasopressin or glutamatergic blockade. These results are consistent with the observation that oxytocin has been shown to sensitize V_1 vasopressin receptors (Poulin et al., 1994) and further suggests that the decrease in blood pressure in the mutant mice engendered by these various antagonists occurs via a common output pathway.

In summary, we have found that functional deletion of the D_5 DAR gene produces hypertension in mice. The elevated blood pressure appears to be attributable to increased sympathetic tone with an involvement of adrenal catecholamines. The exact defect leading to the increase in sympathetic tone is unclear, although it appears to be primarily central in origin. Our current results suggest that D_5 receptor deletion results in an oxytocin-dependent sensitization of V_1 vasopressin and non-NMDA glutamatergic receptor-mediated pathways, potentially within the medulla, leading to increased sympathetic outflow in the mutant mice. This change is not associated with increased synthesis of either oxytocin or vasopressin, and in fact, vasopressin synthesis appears reduced in the D_5 DAR-deficient mice. Furthermore, there is no increase in oxytocin or V_1 vasopressin receptor numbers. This suggests that the increased sensitivity must occur at the level of receptor signaling, possibly via enhanced G-protein interactions, or other regulatory mechanisms, and/or downstream intracellular signaling pathways. The physiological events described here resulting from D_5 DAR deletion may provide new insights into mechanisms for some forms of essential hypertension in humans and may lead to new therapeutic approaches for its treatment.

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