Behavioral/Systems/Cognitive

Physiological Modulation of Intestinal Motility by Enteric Dopaminergic Neurons and the D_2 Receptor: Analysis of Dopamine Receptor Expression, Location, Development, and Function in Wild-Type and Knock-Out Mice

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Dopaminergic neurons are present in both plexuses of the murine bowel and are upregulated after extrinsic denervation but play unknown roles in enteric nervous system (ENS) physiology. Transcripts encoding dopamine (DA) receptors D_1–D_5 were analyzed by reverse transcription-PCR in stomach ≈ duodenum ≈ ileum ≈ proximal ≫ distal colon. Dissected muscle and myenteric plexus contained transcripts encoding D_1–D_3 and D_5, whereas mucosa contained D_1 and D_3–D_5. D_1–D_5 expression began in fetal gut [embryonic day 10 (E10)], before the appearance of neurons (E12), and was sustained without developmental regulation through postnatal day 1. *In situ* hybridization revealed that subsets of submucosal and myenteric neurons contained mRNA encoding D_2 or D_3. Immunoblots confirmed that D_1, D_2, and D_5 receptor proteins were present from stomach through distal colon. Subsets of submucosal and myenteric neurons were also D_1, D_2, or D_3 immunoreactive. When double labeled by *in situ* hybridization, these neurons contained mRNA encoding the respective receptors. Total gastrointestinal transit time (TGTT) and colonic transit time (CTT) were measured in mice lacking D_2, D_3, or D_2 plus D_3. Both TGTT and CTT were decreased significantly (motility increased) in D_2 and D_2 plus D_3, but not D_3, knock-out animals. Mice lacking D_2 and D_2 plus D_3 but not D_3 were smaller than wild-type littermates, yet ate significantly more and had greater stool frequency, water content, and mass. Because motility is abnormal when D_2 is absent, the net inhibitory DA effect on motility is physiologically significant. The early expression of DA receptors is also consistent with the possibility that DA affects ENS development.

Key words: **dopamine receptor; dopaminergic; colon motility; gastrointestinal transit time; enteric nervous system; development**

Introduction

Catecholamines modulate gastrointestinal (GI) motility. Sympathetic nerves release norepinephrine (NE), which inhibits acetylcholine (ACh) release from motor neurons (via α2 adrenoceptors) (Scheibner et al., 2002), evokes IPSPs in submucosal neurons (Hirst and Silinsky, 1975; Frieling et al., 1991; Ren et al., 1999; Wood, 1999), and relaxes smooth muscle (Gershon, 1967). The gut, however, also contains dopamine (DA); moreover, the DA to NE ratio is higher in the bowel than in other sympathetic targets, and the gut contains a high concentration of the specific DA metabolite 3,4-dihydrioxyphenylacetic acid (Eaker et al., 1988). The suggestion from these observations, that DA is an enteric neurotransmitter, has been confirmed recently. Enteric dopaminergic neurons, which express tyrosine hydroxylase (TH) and the dopamine transporter (DAT) but lack dopamine β-hydroxylase, have been identified in mouse, guinea pig (Li et al., 2004), and human (Anlauf et al., 2003).

Although enteric dopaminergic neurons develop perinatally, a subset of the early precursors of enteric neurons (TC cells) transiently contains TH and is catecholaminergic (Cochard et al., 1978; Gershon et al., 1984; Baetge et al., 1990). TC cells depend on the transcription factor Mash1 and give rise to noncatecholaminergic neurons, such as those containing 5-HT (Blaugrund et al., 1996). Whether TC cells secrete norepinephrine or DA is unknown, as is the function of the early catecholamine expression in the developing enteric nervous system (ENS). The potential of catecholamines, such as DA, to influence gut or ENS development remains to be explored.

The function of enteric dopaminergic neurons is not clear. DA relaxes the rat jejunum (Lucchelli et al., 1986, 1990), relaxes smooth muscle isolated from dog colon (Grivegnee et al., 1984), and hyperpolarizes guinea pig submucosal neurons (Hirst and Silinsky, 1975). Electrically induced contractions of mouse colon smooth muscle are small in DAT knock-out mice and restored to normal by the combined inhibition of D_1 and D_2 DA receptors (Walker et al., 2000). Endogenous DA may thus inhibit colonic motility, an effect that is potentiated in the absence of DAT. Previous studies on enteric DA, however, have been indirect, and

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 0270-6474/06/262798-10$15.00/0

Table 1. Sequences of primers

Primers	GenBank accession number	Primer sequence	Primer location in the sequence	PCR program
β-Actin	X03672	Forward: 5′-TGT TTG AGA CCT TCA ACA CC-3′	448–467	94°C 30″ + (94°C 0″ + 57°C 9″ + 72°C 24″) × 40
		Reverse: 5′-CAG TAA TCT CCT TCT GCA TCC-3′	1035–1015	
DAT	AF109391	Forward: 5′-ATC TGC CCT GTC CTG AAA G-3′	405–423	94°C 30″ + (94°C 0″ + 57°C 5″ + 72°C 5″) × 40
		Reverse: 5′-TGG TGA AGG AGG AGA AGA AG-3′	522–503	
D_1	AK044723	Forward: 5′-GTA GCC ATT ATG ATC GTC AC-3′	1138–1157	D + (94°C 30″ + 55°C 45″ + 72°C 30″) × 38 + E
		Reverse: 5′-GAT CAC AGA CAG TGT CTT CAG-3′	1350–1330	
D_2	X55674	Forward: 5′-GCA GCC GAG CTT TCA GAG CC-3′	812–821	D + (94°C 45″ + 64°C 60″ + 72°C 60″) × 38 + E
		Reverse: 5′-GGG ATG TTG CAG TCA CAG TG-3′	1343–1324	
D_3	X67274	Forward: 5′-AGG TTT CTG TCA GAT GCC-3′	772–789	D + (94°C 30″ + 55°C 45″ + 72°C 30″) × 38 + E
		Reverse: 5′-ATT GCT GAG TTT TCG AAC C-3′	1047–1029	
D_4	U19880	Forward: 5′-CAC CAA CTA CTT CAT CGT GA-3′	308–327	D + (94°C 30″ + 58°C 60″ + 72°C 45″) × 38 + E
		Reverse: 5′-AAG GAG CAG ACG GAC GAG TA-3′	700–681	
D_5	AK045456	Forward: 5′-CTA CGA GCG CAA GAT GAC C-3′	593–611	D + (94°C 30″ + 61°C 45″ + 72°C 45″) × 39 + E
		Reverse: 5′-CTC TGA GCA TGC TCA GCT G-3′	952–934	
β3-Tubulin	BC031357	Forward: 5′-TGA TGA CGA GGA ATC GGA AG-3′	1359–1378	D + (94°C 30″ + 63°C 1′ + 72°C 45″) × 31 + E
		Reverse: 5′-CCC GAA TAT AAA CAC AAC CCA G-3′	1681–1660	
Sucrase-isomeltase	XM_143332	Forward: 5′-GTT CGA AGG AGA AGC ACT GG-3′	631–650	D + (94°C 30″ + 63°C 1′ + 72°C 45″) × 23 + E
		Reverse: 5′-TGC GGT AGG TTA GAG CAG GT-3′	964–945	
TH	M69200	Forward: 5′-GCA CAT TTG CCC AGT TCT C-3′	1033–1051	94°C 30″ + (94°C 0″ + 57°C 5″ + 72°C 5″) × 40
		Reverse: 5′-TTT ACA CAG CCC AAA CTC CAC-3′	1148–1128	

D, Denature at 94°C for 2 min; E, extension at 72°C for 5 min; ″, seconds; ′, minutes.

enteric DA receptors have not yet been systematically characterized. DA, moreover, can activate β-adrenoceptors (Grivegnee et al., 1984; Lucchelli et al., 1990; Tsai and Cheng, 1992), which complicates analyses of responses to DA, because exogenous DA or endogenous DA in the absence of DAT reaches ectopic sites that are not accessible to endogenously released DA.

There are five subtypes of DA receptor, which can be grouped into two families: D_1-like family, including D_1 and D_5; D_2-like family, including D_2, D_3, and D_4 (Hartman and Civelli, 1997; Tan et al., 2003). We therefore studied the expression of all dopamine receptor subtypes in adult and fetal mouse gut. Experiments were undertaken to identify DA receptors, to determine their location and oral–anal distribution, and to ascertain which receptors mediate actions of dopaminergic neurons. We also determined when during enteric ontogeny the expression of each subtype of DA receptor begins. This timing provides insight into when developing enteric cells might become DA responsive. Because the D_2 and D_3 receptor subtypes were found in the ENS, the relevance of D_2- and D_3-mediated dopaminergic responses in enteric physiology was investigated by measuring GI motility in transgenic mice lacking one or both of these receptor. These experiments suggest that D_2 receptors inhibit intestinal motility and do so physiologically. Because DA receptors were found in the fetal bowel at a time when the gut contains precursors but no neurons, DA receptors may also be developmentally active.

Materials and Methods

Animals and tissue preparation. Experiments were performed with fetal and adult CD-1 mice (25–30 g; Charles River Laboratories, Wilmington, MA) of either sex and with adult knock-out mice (congenic, C57BL/6) lacking D_2, D_3, or both D_2 and D_3 receptors (Jung et al., 1999). CO_2 inhalation was used to kill adult animals. This procedure was approved by the Animal Care and Use Committee of Columbia University. The brain, stomach, duodenum, ileum, proximal colon, and distal colon were removed from the animals and processed for molecular and histological studies. Fetal mice were removed from pregnant dams at embryonic day 10 (E10), E12, E14, E16, and E18. Newborn mice at postnatal day 1 (P1) were also investigated. The day at which a vaginal plug was found was designated as day 0 of gestation.

RNA extraction and preparation of cDNA. The brain, stomach, duodenum, ileum, proximal colon, and distal colon were collected in PBS (0.9% NaCl in 0.01 M sodium phosphate buffer, pH 7.4), which had been treated with 0.1% diethyl pyrocarbonate (depc-PBS). Fetal gut was collected in ice-cold HBSS and stored in RNA Later (Ambion, Austin, TX). After the wall of each piece of gut was opened, the tissue was cleaned with depc-PBS and transferred to Trizol (Invitrogen, Carlsbad, CA) for extraction of total RNA, which was isolated according to the manufacturer's instructions and stored for further use at −80°C. cDNA was prepared from 3 μg of total RNA by reverse transcription in a 30 μl reaction volume with 0.5 μg of random hexamer primers, 0.5 mM dNTPs, 40 U of Rnasin, and 400 U of Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI).

PCR. Pairs of oligonucleotide primers for amplification of cDNA encoding β-actin, β3-tubulin, dopamine receptors (D_1, D_2, D_3, D_4, D_5), DAT, sucrase-isomaltase, and TH were designed from published mouse cDNA sequences. The programs used for PCR amplification with each primer pair are listed in Table 1. The identities of all PCR products were confirmed by sequence analysis. For this purpose, PCR products were subcloned into pGEM-T Easy vectors (PromegaI) by using the TA-cloning kit (Invitrogen). Inserts in two individual clones were sequenced by the dideoxynucleotide-chain termination method in the DNA facility of Columbia University. The sequences of the PCR products obtained from brain and gut with the indicated primers were found to be identical to those of the appropriate regions of the GenBank sequences of the amplified cDNAs.

Real-time PCR. Real-time PCR was used to quantify mRNA encoding DAT and TH in the ileum of transgenic mice lacking D_2 and their littermates as described previously (Li et al., 2004). The expression of DAT and TH was normalized to that of β-actin, a housekeeping gene that is not thought to be subject to regulation. Transcripts encoding β-actin in samples of mouse gut were first quantified by real-time PCR with the SYBR Green Jumpstart *Taq* ReadyMix (Sigma, St. Louis, MO) using a LightCycler instrument (Roche Diagnostics, Indianapolis, IN). Measurements were obtained by referring to standard curves that were prepared by serially diluting plasmid DNA encoding DAT, TH, and β-actin. The dilutions of β-actin and TH plasmid DNA ranged from 1 pg to 10 ng in five series, each of which covered a 10-fold range. Plasmid DNA encoding DAT was serially diluted from 0.01 to 100 pg, again in five series, each of which covered a 10-fold range.

Amplifications were performed in a final volume of 20 μl of a commercial reaction mixture (Sigma) that contained *Taq*DNA polymerase, reaction buffer, dNTPs in which dTTP is replaced by dUTP, SYBR Green

I dye, and $MgCl_2$. The primers for the amplification of cDNA encoding β-actin, DAT, and TH were used at a final concentration of 0.3 μM. The final concentration of $MgCl_2$ was 4.5 mM for the amplification of β-actin, 8 mM for that of DAT, and 7 mM for that of TH. To this mixture was added 2 μl of either the serially diluted plasmid DNA (standards) or the cDNA prepared from tissue. The standards and the cDNA from tissues were simultaneously subjected to real-time PCR analysis in parallel capillary tubes. The PCRs were performed according to the programs in Table 1. The appearance of double-stranded DNA was quantified by measuring the fluorescence of SYBR Green after each step of elongation. A melting point analysis was finally performed to improve the sensitivity and specificity of amplification reactions detected with the SYBR Green I dye; samples were incubated at 95°C for 0 s, at 67°C for 15 s, and then from 67 to 95°C with a transition rate of 0.2°C/s. Data were analyzed with computer assistance using the LightCycler software.

Immunoprecipitation, gel electrophoresis, and immunoblotting. Tissue was harvested from mouse brain (positive control), stomach, duodenum, ileum, proximal and distal colon, washed with PBS, and homogenized in 300 μl of 50 mM Tris buffer, pH 7.4, containing EDTA (1.0 mM), EGTA (2.0 mM), phenylmethanesulfonyl fluoride (1.0 mM), aprotinin (100 μg/ml), and leupeptin (100 μg/ml) (Li et al., 2004). The homogenate was centrifuged at 10,000 $\times$ *g* for 30 min at 4°C to separate a membrane-containing fraction (pellet) from the cytosol. The membrane-containing fraction was solubilized with Triton X-100 (1%) in the same buffer. An aliquot containing 200 μg of protein was then removed for immunoprecipitation with goat antibodies to the D_2 receptor (Santa Cruz Biotechnology, Santa Cruz, CA). Ten microliters of anti-D_2 were added to yield a total volume of 50 μl. After incubation at 4°C overnight, 20 μl of washed UltraLink Immobilized Protein A/G (Pierce, Rockford, IL) was added, followed by incubation at 4°C with gentle agitation overnight. The reaction mixture was washed, centrifuged at 2000 $\times$ *g* for 2 min, and the supernatant was removed for analysis of D_2 protein by Western blotting. For this purpose, proteins (50 μg) were separated by 8.5% SDS/PAGE. The separated proteins were then electroblotted onto polyvinylidene difluoride membranes and immersed in blocking buffer containing 5% nonfat dry milk in Tris base–sodium chloride buffer (TBS) for 30 min at room temperature (RT). The blot was washed with TBS containing 0.05% Tween 20 (TBST) and finally incubated overnight at 4°C with polyclonal primary antibodies to D_1, D_2, or D_5 (diluted 1:1000 in 3% nonfat dry milk) (Table 2). After washing in TBST, the blot was incubated with goat HRP-labeled secondary antibodies to rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at RT. The blot was finally washed with TBST and developed with a chemiluminescent substrate (Pierce).

Immunocytochemistry. Segments of the ileum were collected in PBS to which the muscle relaxant nicardipine had been added to prevent muscle spasm (10^{-6}M; Sigma) (Li et al., 2004). The contents were flushed out with PBS and the preparations were cut open along the mesenteric border. When whole mounts were to be prepared, the tissue was stretched tautly and pinned flat on balsa wood with the mucosal surface facing down. Specimens were fixed for 2 h at RT with 4% formaldehyde (freshly prepared from paraformaldehyde, pH 7.4) and washed three times with PBS for a total of 30 min. Laminar preparations of longitudinal muscle with adherent myenteric plexus (LMMP) and submucosa [containing the submucosal plexus (SMP)] were obtained by dissection of the gut wall. Tissue to be sectioned was cryoprotected overnight at 4°C with 30% sucrose in PBS containing 0.1% sodium azide. The preparations were embedded in OCT compound (Tissue-Tek; Sakura Finetec, Torrance, CA) and sectioned at 10 μm with a cryostat microtome. Sections were air-dried on slides for 1 h at RT. For immunostaining, sections were permeabilized and blocked by incubation for 30 min at RT with 1% Triton X-100 in PBS containing 10% normal rabbit, goat, or lamb serum, corresponding to the host species used to generate secondary antibodies to prevent nonspecific staining. The whole mounts and sections were incubated with primary antibodies overnight (Table 2), either at RT or at 4°C. For the double labeling of D_2 and D_3, primary antibodies from different species were used (Table 2). After washing with PBS three times for 10 min, the tissue was incubated with appropriate affinity purified species-specific secondary antibodies for 1–2 h. The working concentrations of the secondary antibodies used for immunofluorescence were: goat anti-rat Alexa 594 (1:200; Invitrogen), biotin-labeled donkey anti-rabbit (1:200; Jackson ImmunoResearch, West Grove, PA), donkey anti-goat Alexa 594 (1:200; Invitrogen), biotin-labeled donkey anti-goat (1:200; Jackson ImmunoResearch), and cyanine 3 (Cy3)-labeled streptavidin (1:3000; Jackson ImmunoResearch).

Table 2. Primary antibodies

Antigen	Antibody	Dilution		Source
		Immunocytochemistry	Western blot	
D_1	Rat monoclonal	1:500	1:1000	Sigma
D_2	Rabbit polyclonal	1:800	1:1000	Alpha Diagnostic, San Antonio, TX
D_2	Goat polyclonal	1:800	1:1000	Santa Cruz Biotechnology
D_3	Goat polyclonal	1:300	N/A	Santa Cruz Biotechnology
D_5	Mouse monoclonal	N/A	1:1000	Chemicon, Temecula, CA

N/A, Not applicable.

To increase the detection sensitivity, D_2 and D_3 receptors were also located by using biotinylated secondary antibodies in conjunction with a preformed avidin–biotin complex (Elite kit; Vector Laboratories). Bound antibodies were visualized by using the Vectastain Elite peroxidase kit. Whole mounts of LMMP and SMP of mouse ileum, prepared as described above, were both used. Briefly, preparations were incubated (30 min) with 10% normal serum (of the species in which the secondary antibodies were raised) to inhibit nonspecific staining. The blocked tissue was then incubated overnight with primary antibodies, washed with PBS, and exposed with intervening washes in PBS to the biotinylated secondary antibodies (2 h), 0.3% H_2O_2 in 0.3% blocking sera (5 min), Elite ABC reagent (1 h; Vector Laboratories), and 3′,5′-diaminobenzidine solution (2–10 min) until suitable staining developed.

As a control for the specificity of antibodies to the D_2 and D_3 receptors, attempts were made to immunostain the corresponding receptors in the gut of knock-out animals lacking these receptors.

The immunostained tissue was viewed with a Leica (Nussloch, Germany) DMRXA2 microscope. For epifluorescence, the L5 (Alexa 488, FITC) and M2 (Alexa 594, Texas Red, Cy3) dichroic mirror/filter combinations were used. Fluorescence of the red fluorophores was not visible with the L5 dichroic mirror/filter combination, and fluorescence of the green fluorophores was not visible with the M2 dichroic mirror/filter combination. Images were captured digitally with a cooled CCD camera (QImaging, Burnaby, British Columbia, Canada) operated by a Macintosh computer (Apple Computers, Cupertino, CA) using commercial software (Openlab; Improvision, Coventry, UK). Contrast was adjusted by using the Openlab software, which was also used to analyze double labeling by superimposing images. Bright-field images were captured by using the same microscope, camera, and computer software, except that red–green–blue filters were interposed between the microscope and the camera to permit color imaging. Pictures were sized and assembled using Photoshop 7 (Adobe Systems, San Jose, CA) software for the Macintosh computer.

In situ *hybridization. In situ* hybridization was performed using methods that have been described previously (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, mouse ileum was dissected in ice-cold depc-PBS, stretched, and pinned flat on autoclaved balsa wood. Preparations were fixed on their supports in a freshly made, ice-cold solution containing 4% formaldehyde (freshly prepared from paraformaldehyde) for 1.5 h. After washing with depc-PBST, the preparations were incubated overnight with 30% sucrose/depc-PBS at 4°C, embedded in OCT, and frozen in liquid N_2. Sections (12 μm) were cut at −20°C in a cryostat microtome and thaw-mounted onto Colorfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were air-dried for 20–60 min, rinsed in depc-PBST for 5 min, postfixed with 4% formaldehyde at RT for 15 min, and washed again with depc-PBST to eliminate residual fixative. Sections were then

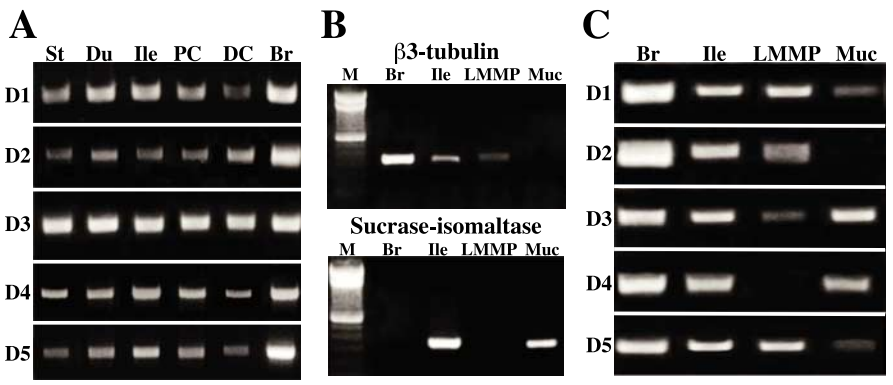

Figure 1. DA receptors are expressed in the gut. Expression of transcripts encoding the dopamine receptors D_1–D_5 was analyzed regionally in the whole gut wall (***A***) and in dissected layers of the wall of the ileum (***B***, ***C***). ***A***, Transcripts encoding each of the five DA receptors were detected in the stomach (St), duodenum (Du), ileum (Ile), proximal colon (PC), and distal (DC) colon. The brain (Br) was investigated as a positive control. ***B***, The presence of transcripts encoding the neural marker β3-tubulin and mucosal epithelial marker sucrase-isomaltase was studied to assess the potential contamination of mucosal preparations with RNA from neurons and LMMP preparations with RNA from the mucosal epithelium. As expected, preparations from Br, whole ileum, and LMMP, but not the mucosa (Muc), contained transcripts encoding β3-tubulin. In contrast, transcripts encoding sucrase-isomaltase were detected in preparations from whole ileum and mucosa but not in those from brain or LMMP. Cross-contamination, therefore, was negligible. M, Molecular marker. ***C***, Transcripts encoding D_1, D_3, and D_5 were present both in the mucosa and the LMMP; those encoding D_2 were present in LMMP but not in mucosa; those encoding D_4 were present in mucosa but not in LMMP.

permeabilized with 1.0 μg/ml proteinase K in depc-PBS, washed with 2.0 mg/ml glycine in depc-PBST, equilibrated in 0.1 M triethanolamine, pH 8.0, for 2 min, acetylated for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine, and washed in depc-PBST. Sections were incubated at 68°C for 1 h in prehybridization buffer, containing 50% formamide (Fisher Scientific), 5× SSC, 5× Denhardt's solution, 0.25 mg/ml yeast tRNA (Roche Diagnostics), 500 μg/ml salmon sperm DNA (Sigma), and 100 μg/ml heparin (Sigma). For hybridization, sections were incubated in the same buffer with 100 ng/ml sense or antisense probes, respectively, for 16 h. The slides were washed in 2× SSC containing 0.1% SDS at RT for 5 min (two times), in 0.1× SSC containing 0.1% SDS at 68°C for 30 min (two times), and in 2× SSC at RT for 5 min. Sections were blocked with 10% goat serum in TBST for 1 h at RT and incubated overnight at 4°C with alkaline phosphatase-labeled Fab fragments of sheep antibodies to digoxigenin (diluted 1:1500; in TBST containing 10% goat serum blocking solution; Roche Diagnostics). After washing in TBST, containing 2 mM levamisole (Sigma) to inhibit endogenous alkaline phosphatase activity, alkaline phosphatase activity was demonstrated with 4-nitroblue tetrazolium according to the directions of the manufacturer (Roche Diagnostics). Sections were coverslipped in a 1:2 mixture of 0.5 M bicarbonate buffer, pH 8.6, and glycerol.

Colon motility. Colon motility was studied in wild-type mice and in littermates lacking D_2, D_3, or both D_2 and D_3 receptors by using previously described methods (Chen et al., 2001). Six mice from each receptor knock-out and from wild-type littermates were used. Briefly, the animals were anesthetized with isoflurane (Baxter Pharmaceutical Products, Deerfield, IL). A glass bead (diameter, 3 mm) was inserted into the colon at a distance of 2 cm from the anal verge. The time required for expulsion of the glass bead was measured and taken as an estimate of colonic motility.

Total GI transit time. These studies were performed in wild-type mice and in littermates lacking D_2, D_3, or D_2 and D_3 receptors by measuring the transit time through the bowel of *Bacillus stearothermophilus* spores (Mathers et al., 1997). Six mice from each genotype were used. The *B. stearothermophilus* spores (Merck, Darmstadt, Germany) were suspended in water at a concentration of $\sim 10^8$ spores/ml. Each mouse received $\sim 2 \times 10^7$ spores in 0.2 ml by gavage. Fecal pellets were collected after 3, 6, 9, 12, 24, 36, 48, 72, and 96 h, vacuum dried overnight, weighed, and ground into a fine powder. The powder was resuspended in water, and 100 μl of the resulting solution was spread on trypticase soy agar plates. Because spores of *B. stearothermophilus* germinate at 65°C, a temperature that is lethal to normal flora, the plates were incubated at 65°C for 12–16 h. The number of *B. stearothermophilus* colonies on each plate was counted, and the mean transit time (MTT) was calculated from the following formula: $\mathrm{MTT}\ (h) = \Sigma\, m_i t_i / \Sigma\, m_i$, where m_i is the number of *B. stearothermophilus* spores passed at time $t_i(h)$ after gavage.

One hour stool collection. Ten mice from each genotype were used for this study. Each mouse was placed in a separate clean cage and observed throughout the 60 min collection period. Fecal pellets were collected immediately after expulsion and placed in sealed (to avoid evaporation) 1.5 ml tubes. Tubes were weighed to obtain the wet weight of the stool, which was then dried overnight at 65°C and reweighed to obtain the dry weight. The stool water content was calculated from the difference between the wet and dry stool weights.

Food and water consumption. Ten mice from each genotype were used for this study. Food and water consumption were studied over the course of a 72 h observation period. Mice were housed separately to permit each animal's food and water consumption to be calculated from the difference in weights of the food and water supply at the beginning and the end of the observation period.

Statistical analysis. Differences between animals of each type were compared by one-way ANOVA.

Results

Transcripts encoding five DA receptors are expressed in the mouse gut

Reverse transcription-PCR was used to investigate the expression of mRNA encoding D_1, D_2, D_3, D_4, and D_5 in the mouse stomach, duodenum, ileum, proximal, and distal colon. Transcripts encoding each of the five DA receptors were found in all of these regions of the bowel and in the brain, which was studied at the same time as a positive control (Fig. 1*A*). To determine the layer of the gut in which these receptors are expressed, the bowel wall was dissected to isolate the mucosa and the LMMP, total RNA was separately extracted from each, and DA receptor expression was again analyzed by reverse transcription-PCR. To evaluate the possibility that the mucosal preparation was contaminated by neurons or the LMMP by mucosa, the presence of transcripts encoding the neuronal marker β3-tubulin and the mucosal epithelial marker sucrase-isomaltase was also investigated. Transcripts encoding β3-tubulin were detected in RNA extracted from the LMMP but not from the mucosa. In contrast, transcripts encoding sucrase-isomaltase were detected in the mucosa but not in the LMMP (Fig. 1*B*). These data indicate that there was no mucosal contribution to RNA from the LMMP or neuronal contamination of RNA from the mucosa. Transcripts encoding D_1, D_2, D_3, and D_5 were detected in RNA extracted from the LMMP, whereas those encoding D_1, D_3, D_4, and D_5 were identified in mucosa (Fig. 1*C*). The observation that transcripts encoding D_2 were only found in the LMMP is consistent with the possibility that the enteric D_2 receptor is neuronal. Similarly, the observation that transcripts encoding D_4 were only observed in the mucosa is consistent with the possibility that the enteric D_4 is non-neuronal. In contrast, both neurons and non-neuronal cells probably express D_1, D_3, and D_5 in the gut.

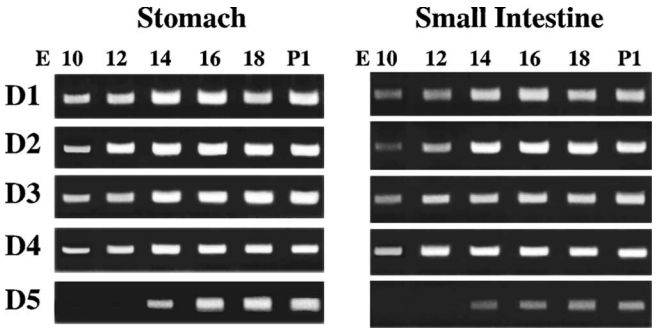

Figure 2. Expression of DA receptors begins early in fetal development and persists in the postnatal gut. DA receptor expression was analyzed by reverse transcription-PCR in the stomach and small intestine from fetal day E10 through postnatal day P1. Transcripts encoding DA receptors D_1–D_4 were detected as early as E10 in both the presumptive stomach and small intestine. Transcripts encoding D_5 were not detected in either organ until E14. Expression of each receptor was detected through P1.

Transcripts encoding DA receptors are expressed in developing mouse gut

Total RNA was extracted from the small intestine and stomach of fetal mice at E10, E12, E14, E16, and E18 and also from postnatal animals at P1 and analyzed by reverse transcription-PCR. Transcripts encoding D_1, D_2, D_3, and D_4 were all detected at each age examined from E10 through P1 in both the small intestine and stomach (Fig. 2*A*,*B*). Transcripts encoding D_5 were not observed until E14 in either the small intestine or the stomach, but once detected at E14, they too persisted through P1 in both regions of the bowel (Fig. 2*A*,*B*). All subtypes except D_5 are thus found in the bowel early in ontogeny. Even D_5 is present while enteric neurons are developing from precursor cells. Receptor expression, however, is not developmentally regulated; expression of all DA receptor subtypes persists in the mature bowel. This pattern is consistent with actions of DA receptors both in the developing and mature gut.

DA receptor immunoreactivities were located in the ENS

Immunoblots were used to confirm that DA receptor proteins, like the previously detected transcripts, are expressed in the mouse gut. Immunocytochemistry was then used to locate the receptors. The immunoreactivities of D_1, D_2, and D_5 (D_3 and D_4 were not examined) were detected by immunoblotting in mouse stomach, duodenum, ileum, proximal, and distal colon (Fig. 3*A*–*C*, respectively). The electrophoretic mobility of each immunoreactive receptor protein was identical to that of the corresponding protein from brain, which was examined as a positive control. Both frozen sections of gut wall (Fig. 4*A*–*C*) and whole mounts of dissected laminar preparations of LMMP and submucosa (Fig. 4*D*–*G*) were used for the immunocytochemical localization of DA receptors. D_1 (Fig. 4*A*), D_2 (Fig. 4*B*,*D*,*F*), and D_3 (Fig. 4*C*,*E*,*G*) immunoreactivities were found in subsets of neurons in both the myenteric and submucosal plexuses.

The locations of transcripts encoding D_2 and D_3 are coincident with their immunoreactivities in the ENS

Double labeling by immunocytochemistry and *in situ* hybridization was used to obtain additional evidence that D_2- and D_3-immunoreactive cells actually express the corresponding receptors. Transcripts encoding D_2 were detected in myenteric neurons of mouse ileum by *in situ* hybridization. Coincident labeling of each of the same cells was found after the preparations were immunostained with antibodies to D_2 (Fig. 5*A*–*D*). Similarly, myenteric neurons of the mouse ileum, in which transcripts encoding D_3 were detected in by *in situ* hybridization displayed coincident labeling after the preparations were immunostained with antibodies to D_3 (Fig. 5*E*–*H*). These observations indicate that subsets of myenteric neurons transcribe and translate D_2 and D_3 receptor proteins. Because D_2 and D_3 are both expressed by enteric neurons, double-label immunocytochemistry (using antibodies that were raised in different species and visualized with contrasting fluorophores) was used to determine whether they are expressed in the same or different neurons. The double labeling in the submucosal plexus of the CD-1 mouse ileum revealed an apparently complete coincidence of expression (Fig. 6*A*–*C*).

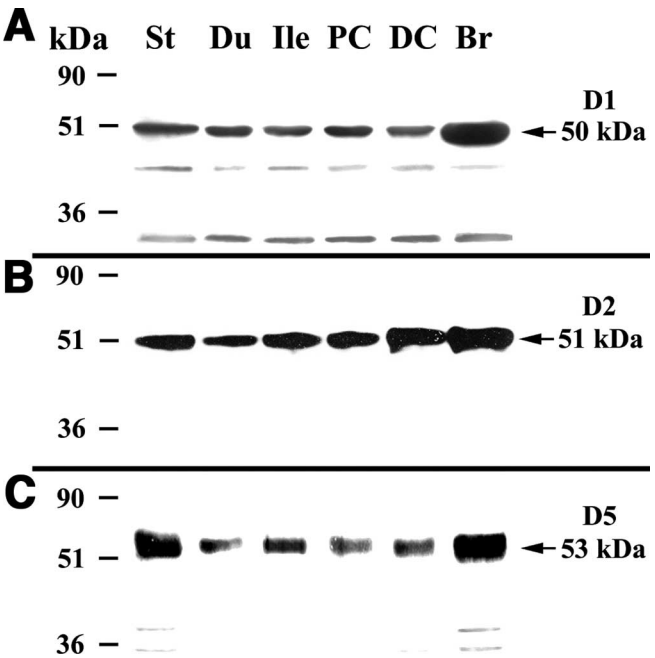

Figure 3. DA receptor immunoreactivity was detected in enteric neurons of mouse ileum. The immunoreactivity was visualized with antibodies to D_1, D_2, and D_3 in both frozen section (***A–C***) and in whole-mount preparations (***D–G***). ***A***, D_1 immunoreactivity is present in the myenteric (MP) and submucosal (SmP) plexuses and in the mucosa (Muc). The arrows indicate the immunoreactive products. ***B, D, F,*** D_2 immunoreactivity is present in subsets of myenteric and submucosal neurons, but not in the mucosa. ***C, E, G,*** D_3 immunoreactivity is present in subsets of myenteric and submucosal neurons. Mucosal immunoreactivity is very weak. Scale bars: (in ***C***) ***A–C***, 5 μm; (in ***G***) ***D–G***, 25 μm.

Control immunocytochemical experiments were performed to determine whether antibodies to the D_2 and D_3 receptors react with gut from transgenic mice that lack these receptors. In contrast to results obtained with wild-type mice, no immunoreactivity was detected when antibodies to D_2 were applied to knock-out mice lacking D_2 (compare Figs. 6*D* and 4*B*,*D*,*F*, 6*A*), and no immunoreactivity was detected when antibodies to D_3 were applied to knock-out mice lacking D_3 (compare Figs. 6*E* and 4*C*,*E*,*G*, 6*B*).

Expression of TH and DAT is increased in the gut of mice that lack D_2

Real-time PCR was used to quantify transcripts encoding TH and DAT in the ilea of transgenic mice lacking D_2 receptors and in those of their wild-type littermates. Expression of TH and DAT in each animal was normalized to that of β-actin and plotted as a ratio. The abundance of transcripts encoding TH was significantly greater in the animals lacking D_2 than in the wild-type mice ($p < 0.05$; $n = 9$). Expression of transcripts encoding DAT

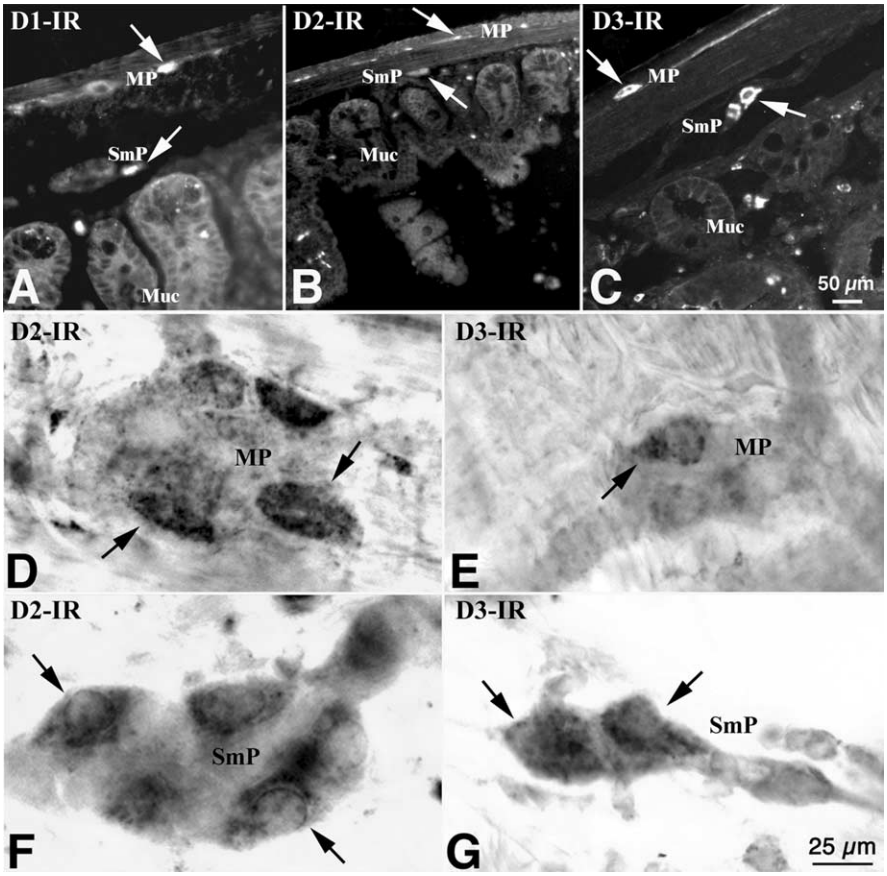

Figure 4. DA receptor immunoreactivity was detected in enteric neurons of mouse ileum. The immunoreactivity was visualized with antibodies to D_1, D_2, and D_3 in both frozen sections (***A–C***) and in whole-mount preparations (***D–G***). ***A***, D_1 immunoreactivity is present in the myenteric (MP) and submucosal (SmP) plexuses and in the mucosa (Muc). The arrows indicate the immunoreactive products. ***B***, ***D***, ***F***, D_2 immunoreactivity is present in subsets of myenteric and submucosal neurons, but not in the mucosa. ***C***, ***E***, ***G***, D_3 immunoreactivity is present in subsets of myenteric and submucosal neurons. Mucosal immunoreactivity is very weak. Scale bars: (in ***C***) ***A–C***, 50 μm; (in ***G***) ***D–G***, 25 μm.

was also significantly elevated in the D_2-deficient animals ($p < 0.01$; $n = 9$). Transcripts encoding TH were >10-fold greater than those encoding DAT. These data suggest that dopamine biosynthesis and reuptake both increase when D_2 receptors are absent.

Propulsive motility is increased in the gut of mice that lack D_2 receptors

The observations that expression of TH and DAT change when D_2 receptors are absent suggests that enteric D_2 receptors and the dopaminergic neurons that activate them are functionally significant. If so, then an abnormality in gastrointestinal physiology would be expected to be present in mice lacking D_2 receptors. We therefore measured total gastrointestinal transit time in animals deficient in D_2, D_3, or D_2 and D_3 (double knock-out) receptors (Fig. 7*A*). D_3 was studied as a reference for D_2 because both receptors are expressed by enteric neurons. Spores of *B. stearothermophilus*, administered by gavage, were used to evaluate transit. Because these spores pass through the entire GI tract and appear in the stool without modification, colony counts as a function of time after gavage can be used to quantify GI transit. The mean GI transit time measured in mice lacking D_2 receptors (12.7 ± 1.4 h) was significantly faster than that measured in their wild-type littermates (18.3 ± 1.4 h; $p < 0.05$; $n = 6$) (Fig. 7*A*). In contrast, the mean GI transit time measured in mice lacking D_3 receptors did not differ significantly from that measured in their wild-type littermates (Fig. 7*A*). Although the mean GI transit time measured in mice lacking both D_2 and D_3 receptors (12.4 ± 0.9 h) was significantly faster than that of their wild-type littermates (17.4 ± 0.9 h; $p < 0.05$; $n = 6$), it did not differ significantly from the mean GI transit time measured in mice lacking D_2 receptors alone (Fig. 7*A*).

Because total GI transit time was abnormal in D_2 receptor-deficient mice, studies were performed to determine whether propulsion is also faster regionally in the colon where abnormalities have previously been reported in mice lacking DAT (Walker et al., 2000). Colonic propulsion was evaluated by measuring the time required to expel a glass bead inserted into the rectum for a distance of 2 cm. Colonic motility was affected by the knock-out of D_2 receptors in a manner that was similar to that of total GI transit (Fig. 7*B*). The time required to expel glass beads from the colon/rectum of mice lacking D_2 receptors (15 ± 5 min) was significantly less than that required by their wild-type littermates (33 ± 4 min; $p < 0.05$; $n = 6$) (Fig. 7*B*). In contrast, expulsion time for the glass beads in mice lacking D_3 receptors did not differ significantly from that measured in their wild-type littermates (Fig. 7*B*). Although the time needed for glass bead expulsion in mice lacking both D_2 and D_3 receptors (16 ± 4 min; $n = 6$) was significantly less than that needed by their wild-type littermates (31 ± 4 min; $p < 0.05$; $n = 6$), it did not differ significantly from that needed by mice lacking D_2 receptors alone (Fig. 7*B*). These data suggest that D_2 plays an important role in the regulation of GI transit and colonic motility.

The decrease in gastrointestinal transit time in mice lacking the D_2 receptor was reflected in corresponding changes in stool frequency, dry weight, and water content, which were determined from fecal pellets obtained during a 1 h collection period (Fig. 8). Stool frequency (Fig. 8*A*), dry weight (Fig. 8*B*), and water content (Fig. 8*C*) were all significantly greater in D_2 knock-out and D_2/D_3 double knock-out mice than in their wild-type littermates. In contrast, the knock-out of D_3 did not cause a significant change in any of these parameters (Fig. 8*A–C*). The effect of the D_2/D_3 double knock-out was similar to that seen in animals in which only D_2 was deleted. The rapid gastrointestinal and colonic transit in D_2 knock-out mice adversely affected the ability of these animals to keep pace with their littermates in growth. Mice lacking D_2 and the double knock-out animals lacking both D_2 and D_3 weighed significantly less than their wild-type littermates (Fig. 8*D*), yet they consumed significantly more food (Fig. 8*E*) and water (Fig. 8*F*). Despite an increased food and water intake, therefore, the D_2 and D_2/D_3 knock-out mice gained weight more slowly than their wild-type littermates. The increased food and water intake of these animals was simply re-

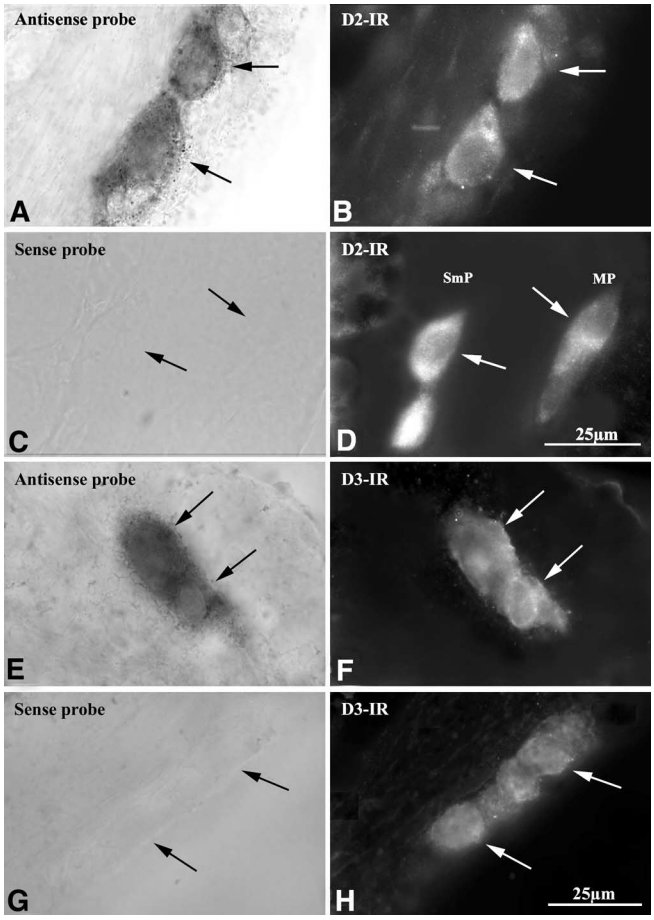

Figure 5. Combined *in situ* hybridization and immunocytochemistry verifies that D_2 and D_3 immunoreactivities (IRs) are found in neurons that coexpress transcripts encoding these proteins. *In situ* hybridization is illustrated in the left panels (***A***, ***C***, ***E***, ***G***), and immunocytochemistry is illustrated in the right panels (***B***, ***D***, ***F***, ***H***). Transcripts encoding D_2 (***A***) and D_2 immunoreactivity (***B***) are found in the same neurons. A sense probe (control; ***C***) does not label D_2-immunoreactive neurons (***D***). Transcripts encoding D_3 (***E***) and D_3 immunoreactivity (***F***) are found in the same neurons. A sense probe (control; ***G***) does not label D_3-immunoreactive neurons (***H***). MP, Myenteric plexus.

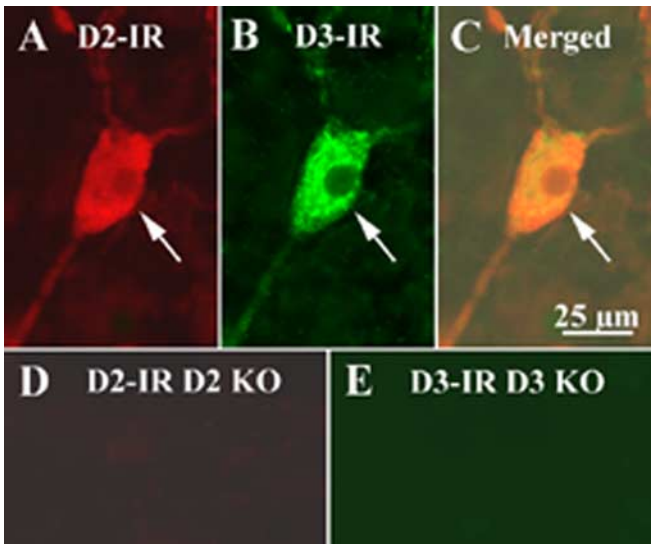

Figure 6. D_2 and D_3 immunocytochemistry were performed on the submucosal plexus of the ileum of CD-1 mice. The same tissue preparation from D_2 and D_3 KO mice was used as control. D_2 receptor immunoreactivity (IR) was revealed by a D_2 rabbit antibody and a donkey anti-rabbit Alexa 594 secondary antibody. D_3 receptor immunoreactivity was revealed by a D_3 goat antibody, a biotinylated donkey anti-goat secondary antibody, and streptavidin FITC. On the tissue of CD-1 mice, D_2-immunoreactive products were present in the enteric neurons (***A***), and D_3-immunoreactive products were also present in the enteric neurons (***B***). D_2 and D_3 immunoreactivities were colocalized in the same cell (***C***). However, D_2 immunoreactivity was not detected on the tissue of D_2 knock-out (KO) mice (***D***); no D_3 immunoreactivity was detected on the tissue of D_3 knock-out mouse (***E***). The arrows indicate the immunoreactive neurons. Scale bar: (in ***C***) 25 μm.

flected in an increased weight and water content of their stool output, suggesting that the fast rate of GI transit in mice lacking D_2 may preclude complete digestion and/or absorption.

Discussion

The current study was undertaken to investigate the physiological significance of the enteric dopaminergic neurons that have been found recently to be present in the mammalian ENS (Anlauf et al., 2003; Li et al., 2004). Although DA can activate adrenoceptors (Lucchelli et al., 1990; Tsai and Cheng, 1992), active dopaminergic neurons would probably be more likely to act physiologically through DA receptors than through adrenoceptors, which evolved to respond to norepinephrine and epinephrine. In fact, some previous studies attributed all of the actions of exogenous DA on the bowel to the nonspecific stimulation adrenoceptors and denied the existence of enteric receptors for DA (Grivegnee et al., 1984; Lucchelli et al., 1990). In contrast to these reports, the current observations suggest that the gut is well endowed with specific DA receptors. Transcripts encoding all five classes of DA receptor were expressed throughout the proximodistal axis of the bowel, and the immunoreactivities of all of these receptors, except D_4, were also found to be present in layers of the bowel that contain neurons. Transcripts encoding D_4 were confined to the mucosal layer, but each of the others potentially mediate dopaminergic neurotransmission. D_1, D_3, and D_5 were expressed both in nerve-containing layers of the gut and in the mucosa; however, both the transcripts encoding D_2 and its immunoreactive protein were restricted to neurons. D_2 is therefore likely to be an important mediator of neuronal responses to DA. This idea was supported by the observation that transcripts encoding TH and DAT were both increased in transgenic mice that lack D_2. Stimulation of D_2 thus seems to be important in feedback regulation of neuronal DA biosynthesis (via TH) and reuptake (via DAT). The upregulation of TH, which should increase DA biosynthesis, may be a compensation for the loss of D_2-mediated effects. The upregulation of DAT may be a compensation for the increased biosynthesis and consequent release of DA.

The phenotype of mice that lack D_2 suggests that D_2 is a major mediator of the effects of endogenous DA in the ENS. Consistent with early observations that DA inhibits subsets of enteric neurons that receive an inhibitory innervation (Hirst and Silinsky, 1975), the knock-out of D_2 was followed by an increase the rate of total gastrointestinal transit and a regional increase in colonic motility. These effects were D_2 specific, in that they were not mimicked by the knock-out of D_3; moreover, the alterations in motility induced by the double knock-out of D_2 and D_3 were indistinguishable from those that followed the knock-out of D_2 alone. Essentially, therefore, these experiments suggest that endogenous DA exerts a net inhibitory effect on intestinal motility and does so primarily via enteric neuronal D_2 receptors. The abnormally rapid transit that follows the knock-out of D_2, moreover, appears to be a severe handicap for mice. The animals ate and drank more than their littermates but still failed to gain weight normally. The excess food and drink lead to increases in the dry weight and water content of stool. One would imagine

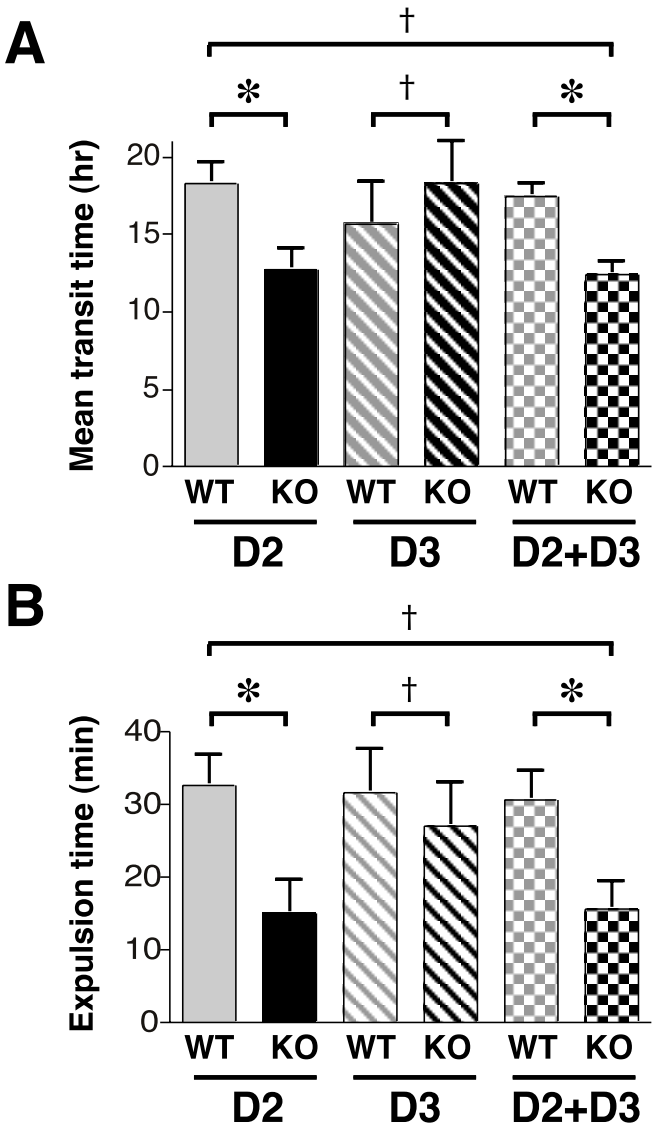

Figure 7. Total gastrointestinal transit time and colonic motility are accelerated in mice that lack D_2 receptors. ***A***, Mean gastrointestinal transit time was measured by using spores of *B. stearothermophilus*. Transit time in mice lacking D_2, D_3, or D_2 and D_3 (double knock-out) was compared with that of wild-type littermates. Relative to wild-type littermates, transit time was reduced (faster) in D_2 knock-out and double knock-out animals; however, transit time in D_3 knock-out mice did not differ from that of their wild-type littermates. Transit time in double knock-out animals was not different from that in mice lacking only D_2. ***B***, Colonic motility was estimated by measuring the time require to expel a glass bead inserted into the rectum for a distance of 2 cm. Relative to wild-type littermates, this time was significantly decreased (faster motility) in mice lacking only D_2 or D_2 plus D_3 but was not different in mice lacking only D_3. The time required to expel the bead in double knock-out mice was not different from that in mice lacking only D_2. $^*p < 0.05$; †, *p* is not significant. KO, Knock-out; WT, wild type. The error bars indicate SEM.

that animals with this problem would do very poorly in the wild where, in contrast to well regulated animal quarters, access to food and water may be problematic.

Inhibition plays critical roles in the regulation of intestinal motility. For example, the ENS is known to exert a tonic inhibitory influence on myogenic pacemakers of the enteric muculature (Wood, 1980). As a result of this constitutive inhibition, the administration of drugs that suppress the output of enteric motor neurons can lead to intestinal spasm and constipation. Inhibition is also critical in the descending relaxation of the peristaltic reflex (Costa and Furness, 1976). Neither inhibition of the tonic inhibitory output of the ENS nor a direct inhibition of the intestinal musculature is likely to be relevant to the effects of D_2 stimulation. The fact that propulsive motility is retained and, in fact, enhanced when D_2 is deleted indicates that coordinated peristaltic reflexes must occur in the mutant animals; the gut is not in spasm and the mice are not constipated. It follows that ganglionic microcircuits, as well as the final common excitatory and inhibitory motor neurons used by the ENS to drive the enteric musculature, must also be functioning and subject to regulation. Synaptic transmission within ganglia and release of ACh, nitric oxide, and other transmitters to smooth muscle thus must occur after the deletion of D_2.

Rapid transit through the total bowel and the colon of mice that lack D_2 suggests that propulsive reflexes occur too often or too powerfully, which implies that the site of D_2-mediated inhibition is within ganglia, affecting the microcircuits that govern peristaltic and/or secretory regulation. This suggestion is consistent with the immunocytochemical location of D_2, which was prominent on the neuritic processes of both myenteric and submucosal neurons. In this location, endogenous DA acting on axonal D_2 receptors is in a position to inhibit the release of ACh and thus to decrease the strength of neurotransmission in prokinetic pathways. In fact, DA has been found to inhibit the evoked release of ^{3}H-ACh from enteric neurons (Kusunoki et al., 1985; Takahashi et al., 1991). This effect, like all of those that are mediated by D_2, involves a pertussis toxin-sensitive G-protein; furthermore, the ability of DA to interfere with ^{3}H-ACh release is blocked by the D_2 antagonist domperidone. Domperidone, moreover, exerts a gastrokinetic effect that has been attributed to its ability to block D_2 receptors (Takahashi et al., 1991). Endogenous DA is released from electrically stimulated neurons of the guinea pig stomach and decreases ACh release (Shichijo et al., 1997). The D_2 antagonists, domperidone, and sulpiride both enhance the release of ACh, suggesting that endogenous DA release provides a constitutive level of inhibition that is relieved by the antagonists. The knock-out of D_2 would be expected to mimic the effects of the D_2 antagonists and increase release of ACh within the ENS.

It is striking that the effects of knocking out D_2 are similar to those exerted on the bowel by 5-HT_4 agonists. Tegaserod, a partial agonist *in vivo* at 5-HT_4 receptors, accelerates gastric emptying and gastrointestinal transit (Degen et al., 2001; Liu et al., 2005). 5-HT_4 receptors, moreover, like D_2, are most concentrated on neurites within enteric ganglia (Liu et al., 2005). Domperidone (Barone, 1999; Drolet et al., 2000) and tegaserod are both used therapeutically as prokinetic agents, although domperidone is often thought to be more effective on the proximal gut (Longo and Vernava, 1993; Jost, 1997) and so has been used to relieve the symptoms of dyspepsia and gastroparesis (Horowitz and Fraser, 1995; Tonini et al., 2004), whereas tegaserod is used to treat chronic constipation and constipation-predominant irritable bowel syndrome (Prather et al., 2000; Muller-Lissner et al., 2001; Johanson, 2004; Galligan and Vanner, 2005). Domperidone and DA, however, have both been shown to exert effects on the colon and rectum as well as on the upper bowel (Wiley and Owyang, 1987), whereas tegaserod accelerates gastric emptying (Degen et al., 2001; James et al., 2004; Crowell et al., 2005) and has also been useful in treating dyspepsia and gastroparesis (Banh et al., 2005; Galligan and Vanner, 2005; Zuberi et al., 2005). The acceleration by D_2 knock-out of total gastrointestinal and colonic motility suggests that D_2 receptors are important at all levels of the gut and are not restricted in their function to the proximal

bowel. This suggestion is consistent with the even distribution of transcripts encoding D_2 and other DA receptors throughout the proximodistal axis of the gut. Inhibition or deletion of D_2 and stimulation of 5-HT_4 receptors, therefore, appear to achieve similar prokinetic effects in both the upper and lower bowel. This would occur if the strength of neurotransmission in prokinetic pathways within the ENS were to be regulated by opposing receptors, D_2 mediating inhibition and 5-HT_4 mediating excitation.

The earliness of the development of enteric DA receptors was unexpected. Except for D_5, all were present by E10. Colonization of fetal mouse gut by émigrés from the neural crest begins at E9.5–E10 (Rothman et al., 1984; Baetge and Gershon, 1989; Young et al., 2003), although enteric neurons cannot be detected until ~E12 (Rothman et al., 1984). Expression of DA receptors, including the neuronally associated D_2 subtype, thus precedes the development of terminally differentiated enteric neurons, although it is coincident with the presence in the gut of crest-derived neuronal/glial precursors. The early expression of enteric DA receptors is consistent with the possibility that DA affects the development of the bowel and/or the ENS. The sustained expression of these receptors suggests that their function is not restricted to development but also involves signaling in the mature gut.

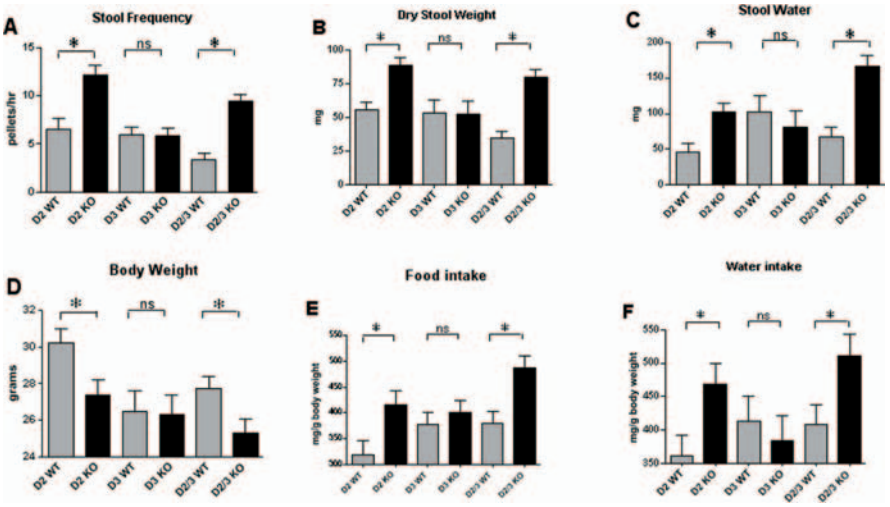

Figure 8. Stool frequency, dry weight, and water content are increased in mice lacking D_2, which are smaller than their wild-type littermates, although they consume more food and water. ***A***, Stool frequency. ***B***, Stool dry weight. ***C***, Stool water content. ***D***, Body weight. ***E***, Food intake. ***F***, Water intake. For each of the parameters measured, wild-type (WT) littermates are compared with mice lacking D_2 (D_2 KO) only, D_3 (D_3 KO) only, or D_2 and D_3 ($D_{2/3}$ KO). *$p < 0.05$. The error bars indicate SEM.

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