Uropathic Observations in Mice Expressing a Constitutively Active Point Mutation in the 5-HT₃ₐ Receptor Subunit

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Mutant mice with a hypersensitive serotonin (5-HT)₃ₐ receptor were generated through targeted exon replacement. A valine to serine mutation (V135S) in the channel-lining M2 domain of the 5-HT₃ₐ receptor subunit rendered the 5-HT₃ receptor ~70-fold more sensitive to serotonin and produced constitutive activity when combined with the 5-HT₃ₐ subunit. Mice homozygous for the mutant allele (5-HT₃ₐ vs/vs) had decreased levels of 5-HT₃ₐ mRNA. Measurements on sympathetic ganglion cells in these mice showed that whole-cell serotonin responses were reduced, and that the remaining 5-HT₃ receptors were hypersensitive. Male 5-HT₃ₐ vs/vs mice died at 2–3 months of age, and heterozygous (5-HT₃ₐ vs/+) males and homozygous mutant females died at 4–6 months of age from an obstructive uropathy. Both male and female 5-HT₃ₐ mutant mice had urinary bladder mucosal and smooth muscle hyperplasia and hypertrophy, whereas male mutant mice had additional prostatic smooth muscle and urethral hyperplasia. 5-HT₃ₐ mutant mice had marked voiding dysfunction characterized by a loss of micturition contractions with overflow incontinence. Detrusor strips from 5-HT₃ₐ vs/vs mice failed to contract to neurogenic stimulation, despite overall normal responses to a cholinergic agonist, suggestive of altered neuronal signaling in mutant mouse bladders. Consistent with this hypothesis, decreased nerve fiber immunoreactivity was observed in the urinary bladders of 5-HT₃ₐ vs/vs compared with 5-HT₃ₐ wild-type (5-HT₃ₐ vs/+) mice. These data suggest that persistent activation of the hypersensitive and constitutively active 5-HT₃ₐ receptor in vivo may lead to excitotoxic neuronal cell death and functional changes in the urinary bladder, resulting in bladder hyperdistension, urinary retention, and overflow incontinence.

Key words: 5-HT₃; mouse; knock-in mutation; bladder; hypertrophy; afferent innervation

Introduction

The serotonin (5-HT)₃ receptor is unique among the 5-HT receptor subtypes because it belongs to the family of excitatory ligand-gated ion channels. Pentamers of 5-HT₃ₐ subunits can form the channel pore, where each subunit has four transmembrane domains, the second (M2) of which lines the ion channel (van Hooft and Yakel, 2003). 5-HT₃ₐ receptors can exist functionally as either homopentameric channels of the 5-HT₃ₐ subunit or as heteromers with the recently cloned auxiliary 5-HT₃B subunit (Davies et al., 1999). However, because the 5-HT₃ₐ subunit alone cannot produce functional receptors, the 5-HT₃ₐ subunit is an essential component of all serotonin-gated ion channels (Dubin et al., 1999; Dang et al., 2000).

5-HT₃ receptors are expressed in both the peripheral and CNS and have been implicated in CNS functions such as cognition, anxiety and emesis, as well as sympathetic, parasympathetic, and sensory functions in the peripheral nervous system (PNS) (Tecott et al., 1993; Jackson and Yakel, 1995; Johnson and Heinemann, 1995; Morales and Wang, 2002). 5-HT₃ receptors are important in nociceptive processing (Alhaider et al., 1991; Eide and Hole, 1993; Zeitz et al., 2002), consistent with expression on primary sensory afferents in the dorsal root ganglion (DRG) and on neurons in the dorsal horn of the spinal cord (Hamon et al., 1989; Kidd et al., 1993, Tecott et al., 1993; Kia et al., 1995; Morales and Wang, 2002; Zeitz et al., 2002). 5-HT₃ receptors on vagal sensory afferents modulate visceral afferent and efﬁent information in the gastrointestinal tract and cardiovascular system (Leslie et al., 1990; Merali et al., 1992; Veelken et al., 1993; Sevoz-Couche et al., 2003). In the enteric nervous system, 5-HT₃ receptors also regulate gut motility and peristalsis (Galligan, 2002). Within the lower urinary tract, presynaptic 5-HT₃ receptors have been implicated in parasympathetic transmission to the urinary bladder through neuronal acetylcholine (ACh) release and smooth muscle contraction (Chen, 1990; Barras et al., 1996).

Various mutations in the M2 domain of the 5-HT₃ₐ nicotinic ACh (nACh), GABA, and glycine receptors have been shown to produce hypersensitivity to agonist stimulation. Some of these mutations occur naturally and are pathologic (Lester and Kar...
schin, 2000), and knock-in mice carrying hypersensitive nicotinic receptor mutations have yielded (patho)physiological insights into nACh receptor function (Lester et al., 2003). To date, there are no known diseases or animal models linked to point mutations of 5-HT<sub>3A</sub> receptors. A valine to serine mutation in the M2 domain of the 5-HT<sub>3A</sub> receptor subunit (V13’S) was previously shown to produce a homomeric receptor ~70-fold more sensitive to 5-HT than the wild-type (WT) receptor when expressed in oocytes (Dang et al., 2000). The purpose of the present study was to further characterize the hypersensitive V13’S mutation in vivo and to evaluate the V13’S mutation as a gain of function alteration in knock-in mice.

Coexpression of the mutant 5-HT<sub>3A</sub> subunit with the wild-type 5-HT<sub>3B</sub> subunit resulted in a 5-HT<sub>3</sub> receptor that was constitutively active in addition to showing hypersensitivity to 5-HT. Introduction of the V13’S mutation into mice by targeted exon replacement resulted in expression of 5-HT<sub>3A</sub> wt<sup>−/−</sup> receptors that were similarly hypersensitive and constitutively active. Unexpectedly, 5-HT<sub>3A</sub> wt<sup>−/−</sup> mice died prematurely from complications related to bladder overactivity, chronic urinary retention, and urinary tract outlet obstruction. Additional characterization of the morphologic, pharmacologic, and cystometric changes in the urinary bladder and urethral outlet tissues of 5-HT<sub>3A</sub> mutant mice showed that many of these changes are characteristic of urinary bladder outlet obstruction (BOO), as seen in patients with benign prostatic hyperplasia (BPH) or neuropathic lesions (Turner and Brading, 1997). These data may reflect an important role of 5-HT<sub>3</sub> receptors in lower urinary tract (patho)physiology.

Materials and Methods

**Targeted exon replacement of the 5-HT<sub>3A</sub> Receptor.** A 6 kb genomic DNA fragment was cloned from a mouse 129Sv/J genomic library (Stratagene, La Jolla, CA) that contained exons 6–9 of the mouse 5-HT<sub>3A</sub> gene. This fragment was cloned into the pGEM5-Zf(+) vector (Promega, Madison, WI), and the M2 channel domain encoded by exon 7 was later used for screening the mutation. The targeting vector was generated by introducing a LoxP-flanked neomycin resistance cassette (Neo), driven by the phosphoglycerate kinase (PGK) promoter, into a SwaI site in intron 5 and a PGK-thymidine kinase (TK) cassette into the 3’ end of the genomic clone. A NotI linearized targeting vector was electroporated into 129Sv/J-derived CJ7 embryonic stem (ES) cells, and homologous recombinant ES clones were double selected in the presence of 180 μg/ml active G418 (Sigma, St. Louis, MO) and 0.2 μM 2′-fluoro-2′-deoxy-1β-D-arabinofuranosyl-5-ido-uracil (FIAU; Sigma). G418 and FIAU resistant ES clones were identified by PCR using a 5′-flanking primer (5E) upstream of the targeting vector sequence (5′-AGCTGCTCCCTCTGATGGCCTAAAGGTCGGTCTGAGGCTTGAGAGTGGCTC-3′) and a 3′ primer (NI) internal to the Neo cassette (5′-GATCAGCCATCTTCTACATATACTATTCTCT-3′). Recombinant ES clones were confirmed by EcoRI Southern blot analysis using both 5′- and 3′-flanking region probes. The 5-HT<sub>3A</sub> wild-type allele contained a 10 kb EcoRI fragment detected by both the 5′- and 3′-probes. Incorporation of the Neo cassette introduced an EcoRI site into the targeted allele, resulting in detection of a 5.5 kb fragment by Southern blot and DNA sequence analysis as described above for ES clone screening, as well as by PCR across the Neo deletion site. This PCR assay used primers ND1 (5′-AATCTAAGAAAAGAACTAGAAGGTTGTGGAAAGG3′) and ND2 (5′-CAAGCTCATACCTCGAAGCAGGATATTGACG-3′) and amplified a 334 bp fragment containing the single LoxP site. All mice used for studies were derived from subsequently established heterozygous (5-HT<sub>3A</sub> wt<sup>+/−</sup>) breeding pairs maintained on a mixed genetic background of 129Sv/J×C57BL/6J×DBA/2. All animal use procedures were overseen and approved by the Roche Palo Alto Institutional Animal Care and Use Committee or the Caltech Institutional Animal Care and Use Committee.

Reverse transcriptase PCR analysis of 5-HT<sub>3A</sub> transcripts in 5-HT<sub>3A</sub> wild-type and mutant mice. Total superior cervical ganglion (SCG) RNA was isolated using the RNAzol B reagent (Tel-Test, Friendswood, TX) according to supplier specifications. RNA samples were diluted 1:100, and reverse transcriptase (RT)-PCR was performed using 1 μl of serial dilutions of RNA as template in a 25 μl reaction using the Thermostep One-Step RT-PCR kit (Invitrogen, Rockville, MD). The primers used for amplification of the 5-HT<sub>3A</sub> receptor were VS1 and VS2 (described above), and the primers used for amplification of the α7 nACh receptor internal control were 7-1 (5′-GATCATTGTCTGAGGCTTGAGATCAGCAGCAGAAGGAG3′) and 7-2 (5′-GATCATCTGCACAGGATATTGACGATTGACG-3′). Both sets of primers were amplified simultaneously in the PCR to generate amplicons of 300 and 700 bp for the 5-HT<sub>3A</sub> and α7 nACh receptors, respectively.

Quantitation of mRNA derived from mutant or wild-type alleles was estimated from the relative representation of the mutant and wild-type sequences obtained by sequencing the RT-PCR products derived from 5-HT<sub>3A</sub> wt<sup>−/−</sup> mice. This approach is based on the premise that although automatic DNA sequencing signals are highly variable at individual nucleotide positions, these signals retain their relative sizes within a given stretch of sequence when compared among DNA samples sequenced under identical conditions. In comparing the relative signals from the mutation to nearby constant nucleotides between wild-type and mutant sequences, one can estimate the relative amount of mutant versus wild-type mRNA in heterozygous mice. After sequencing of the RT-PCR products from 5-HT<sub>3A</sub> wt<sup>−/−</sup>, 5-HT<sub>3A</sub> wt<sup>−/−</sup>, and 5-HT<sub>3A</sub> wt<sup>−/−</sup> mice using primer VS3, the area under the wild-type and mutant peaks was normalized to the 3T and 1A nucleotide positions (numbered with respect to the wild-type V13’ codon GTC). The peak area ratios 1G/−3T and 1G/−1A in each sample was defined as 100%. Similarly, the normalized signals from 1T, 2C, and 3A (TCA serine codon) were used to estimate the amount of mutant sequence (the 5-HT<sub>3A</sub> wt<sup>−/−</sup> sample was defined as 100%). Similarly, the normalized signals from 1T, 2C, and 3A (TCA serine codon) were used to estimate the amount of mutant sequence (the 5-HT<sub>3A</sub> wt<sup>−/−</sup> sample was defined as 100%). Using this approach, average estimates were made of the amount of mutant and wild-type mRNA expressed in 5-HT<sub>3A</sub> wt<sup>−/−</sup> mice.

Xenopus oocyte electrophysiology. As described previously (Dang et al., 2000), cDNA clones for the mouse 5-HT<sub>3A</sub> subunit (Marić et al., 1991) and for the human 5-HT<sub>3B</sub> (DNase (Davis et al., 1999) were subcloned into the oocyte expression vector plasmid pAMV (Nowak et al., 1995). Mutations in the cDNA were made using the Quick-Change mutagenesis
kit (Stratagene). Plasmids were linearized with NcoI and used as template to produce mRNA using the T7 mMESSAGE mMACHINE kit (Ambion, Austin, TX). Stage V-VI Xenopus oocytes were harvested and injected with 50 nl of cRNA per oocyte (−0.5 ng/oocyte). The ratio between the 5-HT3A and the 5-HT3B mRNA was 1:1. Two-electrode voltage-clamp recordings were performed 24–36 hr after injection using a GeneClamp500 circuit, a Digidata 1200 digitizer, and pClamp software (Axon Instruments, Union City, CA). The recording solution contained (in m M): 96 NaCl, 2 KCl, 2 MgCl2, 5 HEPEs, pH 7.4 (ND 96). Whole-cell current responses to various drug concentrations at indicated holding potentials (typically −60 mV) were fitted to the Hill equation, $I/I_{\text{max}} = 1/(1 + (EC_{50}/[A])^n)$, where $I$ is agonist-induced current at concentration [A]; $I_{\text{max}}$ is the maximum current, $EC_{50}$ is the concentration inducing half-maximum response, and $n$ is the Hill coefficient.

Primary cell culture and electrophysiology. SCG neurons were dissected from 1- to 3-d-old mice and digested in Ca2+, Mg2+-free Hank’s saline solution containing 0.25% trypsin for 10 min. Cell suspensions were washed and gently triturated, followed by plating onto culture dishes precoated with polyornithine and laminin in MEM supplemented with solution containing 0.25% trypsin for 10 min. Cell suspensions were removed from the culture dishes and injected into Krebs’ buffer. Tissues were mounted between two parallel plate electrodes in thermostatically controlled (37°C) organ baths (10 ml) containing Krebs’ buffer, gassed continuously with 95% O2 and 5% CO2. The composition of the buffer was (in m M): 118.2 NaCl, 4.6 KCl, 1.6 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 10 dextrose, 24.8 NaHCO3. The detrusor strips were equilibrated at a resting tension of 0.5 gm for 1 hr with intermittent washing, followed by a KCl (67 m M) prime. KCl-induced contractions were used to normalize the data to control for differences in absolute contractile values resulting from differences in tissue size or health. Changes in isometric force were measured by Grass FTO3c transducers (Grass Instruments, Quincy, MA) and digitized using MacLab data acquisition software (ADInstruments, Colorado Springs, CO). To measure neurogenically-mediated contraction, a train of square electrical pulses was applied for 5 sec with pulse frequency increasing in twofold increments (10 V with a pulse width of 0.5 msec). Pulses were delivered by a Grass S88 stimulator and divided across the tissue baths using a MedLab Stimulator (Grass Telefactor, West Warwick, RI). Electrically induced contractions were confirmed to be neurogenically mediated by their sensitivity to tetrodotoxin (100 m M). Neurogenic contractions were plotted by fitting a nonlinear equation. In experiments where carbachol was used to induce contraction, cumulative addition of the drug was applied (van Rossum, 1963). The potency of carbachol was determined by fitting mean contractions for each concentration of the drug to the following equation, which is percentage contraction = [Carbachol]$^{2}3[H]/EC_{50}$ + (Carbachol)$^{2}3[H]$

### Urethral compliance measurements

Biomechanical study of the mouse urethra was performed as described previously (Jankowski et al., 2004). Female mice were anesthetized with halothane and catherized (PE-10) through the urethra. The catheter contributed to the maintenance of urethral length after excision of the urethra and bladder. Specimens were mounted on an ex vivo testing system (37°C; 95% O2, 5% CO2). Briefly, the system consisted of a hydrostatic reservoir attached to a graduated ring stand for controlled application of intraluminal pressure (measured via a strain gauge pressure transducer connected to a pressure monitor) and a laser micrometer for accurate measurement of outer diameter. Pressure and diameter data were continuously recorded via analog-to-digital conversion to a personal computer. Urethral specimens were preconditioned from 0 to 6 mmHg (each pressure held for 10 sec for 10 cycles), followed by pressure increments of 2 mmHg up to 20 mmHg. The experiment was repeated in random order for three positions along the length of the urethra: proximal (a distance of 30% in vivo length from proximal end), middle (50% in vivo length from proximal end), and distal (70% in vivo length from proximal end). Using the pressure and diameter data recorded, compliance (C) and β stiffness (β) values were calculated for each position.

**Compliance (C)**: 

$$C = \frac{(P_{\text{max}} - P_{\text{min}})D_{\text{max}}}{(P_{\text{max}} - P_{\text{min}})D_{\text{max}}}$$

**β Stiffness (β)**: 

$$\beta = \frac{L}{D_{\text{max}} - P}$$

**Mouse cystometry.** Mice were anesthetized with isoflurane and cystometry was conducted as described previously (Cockayne et al., 2000). Briefly, the bladder was exposed through a midline abdominal incision. A saline-filled PE-10 cannula with an enlarged tip was inserted into the dome of the bladder and secured to the bladder with 5-0 Tevdek suture. The cannula was tunneled subcutaneously to the back, exteriorized, tied off, and secured to the skin with 4-0 silk. The muscle layer was closed with 4-0 silk, the skin incision was closed, and the exteriorized part of the catheter was placed into the subcutaneous space. Mice were returned to normal caging for 7 d of recovery. For conscious cystometry, each mouse was placed in a restraint box within a metabolic cage. The bladder cannula was exteriorized and connected to a pressure transducer and infusion pump via a three-way connector. Normal saline was infused into the bladder at a constant rate of 3 ml/hr, and bladder pressure and accumulated void volume was recorded. For carbachol-induced bladder contractions, carbachol or vehicle was administered intravesically as a 0.1 ml bolus.

In vitro pharmacology. Isometric smooth muscle contraction was studied in bladder detrusor strips as described previously (Martin et al., 2000). Briefly, bladders were removed from mice and dissected into whole bladder sections. Isolated bladders were opened by cutting the tissue from the dome of the bladder and secured to the bladder with 5-0 Tevdek suture. The catheter was exteriorized and connected to a pressure transducer and infused via a three-way connector. Normal saline was infused into the bladder at a constant rate of 3 ml/hr, and bladder pressure and accumulated void volume was recorded. For carbachol-induced bladder contractions, carbachol or vehicle was administered intravesically as a 0.1 ml bolus.
Compliance is a measure of the distensibility of the tissue and provided the relative change in diameter per unit change in pressure. $P_{\text{max}}$ and $P_{\text{min}}$ represent the maximum and minimum pressures over the pressure range of interest, and $D_{\text{max}}$ and $D_{\text{min}}$ are the respective diameters measured at these pressures. $\beta$ stiffness is a parameter that has been widely used to quantify the nonlinear elastic properties of tubular biological tissue. It assumes an exponential relationship of the pressure and diameter, with $\beta$ being the exponent coefficient. $P_s$ is an arbitrary reference pressure taken here as 10 mmHg, and $D_s$ is the corresponding diameter measured at that pressure. One-way ANOVA was used for statistical analysis with Student–Newman–Keuls post hoc testing.

Results

In vitro characterization of the hypersensitive 5-HT$_{3A}$ V13'S mutation in Xenopus oocytes

We previously used the Xenopus oocyte expression system to characterize homomeric 5-HT$_{3A}$ receptors containing the V13’S mutation in the M2 channel domain (Dang et al., 2000). The mutant receptor showed slower activation and desensitization kinetics (Dang et al., 2000) (Fig. 1A) and was ~70-fold more sensitive to serotonin than the wild-type 5-HT$_{3A}$ receptor (5-HT$_{3A}$/WT) (Fig. 1B).

In the present study, we performed oocyte expression studies to further characterize 5-HT$_{3A}$ receptors containing the V13’S mutation and to predict the functional properties of homozygous and heterozygous 5-HT$_{3A}$ receptors likely to be expressed in 5-HT$_{3A}$/ knock-in mice. Consistent with previous findings, the homomeric V13’S receptor showed higher spontaneous activation in the absence of agonist, manifested as a slightly increased leak current under voltage clamp that is blocked by the channel blocker 8-[(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8; 100 µM) (Dang et al., 2000) (Fig. 1C). This spontaneous activity represents <10% of the maximal response. We next assessed heterozygous V13’S/5-HT$_{3A}$/WT pentameric receptors by co-injecting a 1:1 mixture of V13’S and 5-HT$_{3A}$/WT cRNAs into oocytes. The V13’S/5-HT$_{3A}$/WT response waveforms desensitized more slowly than the 5-HT$_{3A}$/WT receptor and were comparable with the V13’S/V13’S waveforms (Fig. 1A). The V13’S/5-HT$_{3A}$/WT dose–response relationship was located between the V13’S and 5-HT$_{3A}$/WT, was shallower than either of the former two relationships, appeared to consist of at least two components, and displayed a half-maximal concentration ~10-fold lower than the EC$_{50}$ for the wild-type 5-HT$_{3A}$ receptor (Fig. 1B).

While our efforts in generating the 5-HT$_{3A}$/ knock-in mice were in progress, the human 5-HT$_{3B}$ subunit cDNA was cloned (Davies et al., 1999), and functional analysis suggested that some native 5-HT$_{3}$ receptors are heteromultimers containing at least the 5-HT$_{3A}$ and 5-HT$_{3B}$ subunits. The 5-HT$_{3B}$ subunit alone shows no response to serotonin when expressed in oocytes but modifies serotonin responses when coexpressed with the 5-HT$_{3A}$ subunit. Unexpectedly, when V13’S 5-HT$_{3A}$ and 5-HT$_{3B}$ subunit cRNAs were co-injected into oocytes, the resultant receptor (V13’S/5-HT$_{3B}$) showed high levels of TMB-8-sensitive spontaneous activation (Fig. 1D) that was >50% of the maximal response. The waveforms of additional 5-HT$_{3}$-evoked currents at V13’S/5-HT$_{3B}$ receptors showed more rapid activation and desensitization kinetics than the V13’S/V13’S or V13’S/5-HT$_{3A}$/WT waveforms (Fig. 1D). The additional 5-HT$_{3}$-evoked currents at the V13’S/5-HT$_{3B}$ receptor displayed dose–response relationships with an EC$_{50}$ of ~0.02 nM, resembling those of the V13’S receptor (Fig. 1E). The 5-HT$_{3A}$/WT/5-HT$_{3B}$ receptor displayed concentration–response relationships like those of the homomeric 5-HT$_{3A}$/WT receptor, with an EC$_{50}$ of ~2 nM (Fig. 1E). Therefore, the most striking effect of these in vitro studies is the high level of spontaneous activity in the heteromultimeric V13’S/5-HT$_{3B}$ receptor.

Generation of 5-HT$_{3A}$ receptor knock-in mice

The V13’S valine to serine mutation in the 5-HT$_{3A}$ receptor was introduced into the mouse genome by the targeted exon replacement strategy shown in Figure 2. Heterozygous 5-HT$_{3A}$+/S mice appeared outwardly normal and bred to produce 5-HT$_{3A}$/+,-, 5-HT$_{3A}$/+,-, and 5-HT$_{3A}$/S mice. Mice carrying the homozygous V13’S knock-in mutation were viable and seemed to develop normally to 2 months of age with no adverse clinical signs or altered behavior. However, as shown in Table 1, male homozygous mutant mice, and to a lesser extent homozygous females and heterozygous males and females, died prematurely (Table 1).
expression from the V13’s mutant allele. To address this directly, we measured 5-HT$_{3A}$ mRNA expression by RT-PCR using serial dilutions of total SCG RNA and primers VS1/VS2 for 5-HT$_{3A}$ receptor mRNA and primers a7-1/a7-2 for a7 nACh receptor mRNA as an internal control (Fig. 3A). RT-PCRs from 5-HT$_{3A}$ vs/vs mice had more RNA loading than those from 5-HT$_{3A}$/+ and 5-HT$_{3A}$+ mice as judged by the relative intensities of the a7 band (Fig. 3A). Despite these RNA loading differences, 5-HT$_{3A}$ mRNA signals from 5-HT$_{3A}$+/+ and 5-HT$_{3A}$ vs/+ mice showed little difference, whereas in 5-HT$_{3A}$ vs/vs mice, the 5-HT$_{3A}$ bands were substantially less intense (<25%) compared with those seen in the other two genotypes.

Reduced expression of 5-HT$_{3A}$ mRNA in homozygous mu-

<table>
<thead>
<tr>
<th>5-HT$_{3A}$</th>
<th>Sex</th>
<th>Mortality (dead/total)</th>
<th>Mean lifespan of dead (d)</th>
</tr>
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<tbody>
<tr>
<td>+/+</td>
<td>Male</td>
<td>0/34 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>vs/+</td>
<td>Male</td>
<td>42/144 (29%)</td>
<td>185 ± 11</td>
</tr>
<tr>
<td>vs/vs</td>
<td>Male</td>
<td>33/37 (89%)</td>
<td>71 ± 5</td>
</tr>
<tr>
<td>+/+</td>
<td>Female</td>
<td>0/43 (0%)</td>
<td>NA</td>
</tr>
<tr>
<td>vs/+</td>
<td>Female</td>
<td>15/113 (13%)</td>
<td>209 ± 24</td>
</tr>
<tr>
<td>vs/vs</td>
<td>Female</td>
<td>8/43 (19%)</td>
<td>137 ± 23</td>
</tr>
</tbody>
</table>

NA, Not applicable.
*Observations made over a period of ~400 d.
alleles by estimating the relative representation of mutant and wild-type sequences in SCG neurons of 5-HT3A vs/vs mice. Whole-cell patch clamp analysis on SCG neurons isolated from 5-HT3A vs/vs mice...showed positive over successfully patched neurons for each genotype is shown below each panel.

Figure 4. Serotonin-induced whole-cell currents from SCG neurons of 5-HT3A vs/+ vs/vs mice. Whole-cell patch clamp recordings were obtained from primary cultures of SCG neurons. Representative voltage-clamped current responses from 5-HT3A vs/+ (left panel), 5-HT3A vs+ (middle panel), and 5-HT3A vs+ (right panel) mouse to low (A) and high (B) concentrations of serotonin are shown. The low concentration of serotonin is 0.3 μM, whereas the high concentration is 10 μM in all three. The number of 5-HT3A receptors positive over successfully patched neurons for each genotype is shown below each panel.

of those seen at 10 μM (Fig. 4B, right panel). In contrast, 5-HT at 0.3 μM failed to activate currents in 5-HT3A vs/+ or 5-HT3A vs+ SCG neurons (Fig. 4A, left and middle panels, respectively). The small responses seen in 5-HT3A vs+ neurons displayed both concentration dependence and kinetic characteristics predicted from oocyte expression studies (Fig. 1) and confirm that the 5-HT3A V13′S receptor expressed in vivo is hypersensitive to 5-HT.

Decreased lifespan in 5-HT3A vs+ mice resulting from lower urinary tract dysfunction: pathological evidence

Male homozygous mutant mice died at 2–3 months of age (Table 1). Female homozygous mutant mice, followed by male and female heterozygous mice, also had a decreased lifespan compared with 5-HT3A vs+ mice (Table 1). Moribund 5-HT3A vs+ mutant mice were cachectic and had elevated blood urea nitrogen and proteinuria. Renal pelvic dilatation resulting from urinary tract obstruction and urinary tract bacterial infections was present, as well as pyelonephritis and tuberulointerstitial nephritis (Fig. 5, compare H and G). This was likely the main cause of death in 5-HT3A vs+ mice. Both male and female 5-HT3A vs+ mice had hypersensitive urinary bladders characterized by epithelial and detrusor smooth muscle hyperplasia and hypertrophy (Fig. 5, compare B and D with A and C). Urinary bladder hypertrophy was evident by 6 weeks of age in male and female 5-HT3A vs+ mice, with significant increases in urinary bladder weight and twofold to threefold increases in urinary bladder to body weight ratios (Table 2). In addition to changes in the urinary bladder, male 5-HT3A vs+ mice also had hyperplasia of prostatic urethral epithelium and surrounding smooth muscle (Fig. 5F) and extensive suppurative glandular and periglandular inflammatory cell infiltration of the prostate and seminal vesicles (Fig. 5, compare F and E). Proteinaceous plugs were often found in the bladder neck and proximal urethra of 5-HT3A vs+ males. In some mutant mice, we also observed bone marrow hyperplasia and generalized lymphoid atrophy, likely attributed to systemic stress and bacterial infection.

Pathophysiology associated with lower urinary tract organs in 5-HT3A vs+ mice

Urodynamic studies were performed in 5-HT3A vs/+ vs/vs, and 5-HT3A vs+ mice to determine how histopathological changes observed in 5-HT3A vs+ mice influenced urinary bladder function. Figure 6 shows representative filling cystometrograms from conscious mouse cystometry studies in which voiding reflexes were measured in response to a continuous intravesical infusion of saline. In contrast to 5-HT3A vs+ mice, where normal micturition contractions were observed (Fig. 6, top panel; Table 2), 5-HT3A vs mice did not generate voiding contractions (Fig. 6, bottom panel; Table 3). All 5-HT3A vs mice studied had a consistent phenotype of overlow incontinence that manifested itself as constant urine dribbling (Fig. 6, Table 3, c.d.). Heterozygous mice had an intermediate phenotype that ranged from decreased void intervals and void volumes to dribbling incontinence (Table 3). Similar cystometric and histopathological changes were present in both 8- and 12-week-old mice. In separate experiments where natural voiding behavior was measured in metabolic chambers, 5-HT3A vs mice also had a phenotype of constant dribbling and lacked normal voids compared with 5-HT3A vs+ littermate controls (data not shown).

To determine whether detrusor smooth muscle responses were altered in 5-HT3A vs mice, we measured neurogenic contraction of bladder strips from 6-, 8-, and 12-week-old mice (Fig. 7). Electrical field stimulation of detrusor strips from...
loss of neurogenic-mediated contraction (Fig. 7), carbachol-induced concentration-dependent contractions of the detrusor smooth muscle in both tissue bath studies in vitro (Fig. 8A,B) and in conscious cystometry where carbachol was administered intravesically (Fig. 8C,D) after establishment of baseline voiding cystometrograms (Fig. 6). No differences were seen in either study between wild-type and mutant mice in terms of the efficacy or potency of carbachol on detrusor smooth muscle responses, except at the two highest doses of carbachol in 12-week-old mice in vivo. Together, these data suggest that the V13’s mutation had little or no overall effect on cholinergic receptor function in the urinary bladder.

**Decreased nerve fiber density in the urinary bladder urothelium of 5-HT$_{3A}$ knock-in mice**

The loss of neurogenic bladder contractions in 5-HT$_{3A}$ mutant mice could be attributable to changes in the neuronal innervation of the urinary bladder. 5-HT$_{3}$ receptors are found on DRG sensory afferents and on parasympathetic efferents innervating the urinary bladder, and excitotoxic cell death of 5-HT$_{3A}$ V13’S-expressing neurons could lead to a loss of neurons innervating the lower urinary tract. To address this question, we performed whole-mount immunostaining of the bladder urothelium from 5-HT$_{3A}$ $^{+/+}$ and 5-HT$_{3A}$ $^{vs/vs}$ mice using Substance P immunoreactivity as a marker of primary sensory afferents and PGP 9.5 immunoreactivity as a pan-neuronal marker (Navarro et al., 1997) for all neurons. Consistent with previous observations (Gabella and Davis, 1998), we detected a greater density of nerve fiber innervation in the neck of the urinary bladder compared with the dome in wild-type mice (Fig. 9). In 5-HT$_{3A}$ $^{vs/vs}$ mice, staining for both Substance P and PGP 9.5 was markedly decreased compared with that seen in 5-HT$_{3A}$ $^{+/+}$ controls. This observation is consistent with the loss of functional responses of the detrusor to nerve-mediated stimulation (Fig. 7) and suggests that a disruption of neuronal networks or connectivity may play a role in the altered lower urinary tract physiology seen in 5-HT$_{3A}$ $^{vs/vs}$ mice.

**Assessment of urethral compliance in 5-HT$_{3A}$ $^{vs/vs}$ mice**

To determine whether changes in urethral tone contributed to the overflow incontinence seen in 5-HT$_{3A}$ mutant mice, we measured the biomechanical properties of urethral compliance and β stiffness. Figure 10 shows low pressure (0–6 mmHg)-induced changes in urethral compliance and β stiffness in 5-HT$_{3A}$ $^{+/+}$ and 5-HT$_{3A}$ $^{vs/vs}$ mice, demonstrating that no differences were observed in either of these measures. As expected for this system (Jankowski et al., 2004), the proximal urethra was significantly more compliant than the middle and distal portions when subjected to low-pressure stimulations, and this was similar for both 5-HT$_{3A}$ $^{+/+}$ and 5-HT$_{3A}$ $^{vs/vs}$ mice (Fig. 10A). Although differences in β stiffness were not observed between 5-HT$_{3A}$ $^{+/+}$ and 5-HT$_{3A}$ $^{vs/vs}$ mice for the proximal or middle portions of the urethra, the data did reveal a trend ($p < 0.1$) in which the distal portion of the urethra in 5-HT$_{3A}$ $^{vs/vs}$ mice showed less β stiffness compared with wild-type controls over the entire pressure range tested (Fig. 10B for 0–6 mmHg) (data not shown).

**Discussion**

In this study, we demonstrated that a V13’S gain of function mutation in the 5-HT$_{3}$ receptor subunit resulted in a hypersensitive and constitutively active ion channel, and that expression of this receptor in gene knock-in mice resulted in lower urinary tract dysfunction with overflow incontinence.
active 5-HT₃ receptors in neurons of knock-in mice, these data are consistent with the idea that 5-HT₃A+/– mice expressed a gain of function mutation with a high level of constitutive activity, leading to neurotoxic cell death, and low levels of a hypersensitive receptor in the remaining 5-HT₃A-positive neurons.

Phenotypic characterization of 5-HT₃A mutant mice revealed that both males and females developed severe lower urinary tract dysfunction characterized by enlarged urinary bladders with hyperplasia and hypertrophy of the epithelium and detrusor smooth muscle. These bladders showed loss of responsiveness to neurogenic stimulation with little or no change in responsiveness to an exogenous muscarinic agonist, decreased urinary bladder innervation, and a complete loss of micturition contractions with constant urine leakage and overflow incontinence. In addition to changes in the urinary bladder, male 5-HT₃A+/+ mice had hyperplasia of prostate urethral epithelium and surrounding smooth muscle, with inflammatory cell infiltrate of the prostate and seminal vesicles. The main cause of death in 5-HT₃A mutant mice was likely urinary tract bacterial infection and renal failure resulting from chronic urinary retention and urinary tract obstruction. 5-HT₃A+/– males died more prematurely than females, suggesting that changes in the prostate and surrounding tissues may have exacerbated a common underlying pathology present in both sexes, possibly by creating a greater degree of outlet obstruction. The effect of the V13’S mutation was also highly penetrant in that heterozygotes showed a loss of responsiveness to neurogenic stimulation and an average lifespan between that of homozygous mutants and wild-type controls. Despite the well described role for 5-HT₃ receptors in gastrointestinal, cardiovascular, and CNS functions, we did not observe histopathological changes or overt behavioral signs of dysfunction in these physiological systems in 5-HT₃A+/– mice. Future studies exploring these physiological pathways would be of interest.

Many of the morphological and functional changes in the urinary bladders of 5-HT₃A+/– mice are similar to the changes described for animals or humans with urinary bladder outlet obstruction or neuropathic lesions (Turner and Brading, 1997; Bassuk et al., 2000; Pandita et al., 2000). This raises the question of whether 5-HT₃A+/– mice offer a genetic model for bladder instability associated with partial outlet obstruction or changes in the neuronal control of micturition as seen in neuropathic disease. Partial outlet obstruction in surgically obstructed animals or secondary to BPH is often associated with bladder hypertrophy, detrusor instability, and urinary retention (Hines, 1996; Turner and Brading, 1997). Adaptive changes in the bladder can also occur in response to changes in innervation, and partial denervation of the detrusor has been associated with bladder instability in outflow obstruction, neuropathic disease, and idiopathic

Previous studies demonstrated that the V13’S mutation in the A subunit of the 5-HT₃ receptor rendered the receptor hypersensitive to 5-HT (Dang et al., 2000). These findings have been extended to show that coexpression of the V13’S 5-HT₃A subunit with the wild-type 5-HT₃A subunit (Davies et al., 1999) resulted in a constitutively active V13’S/5-HT₃A heteromultimeric receptor with 5-HT potency similar to that of the homomeric V13’S receptor. Because 5-HT₃ subunits can form homomeric 5-HT₃A and heteromeric 5-HT₃A/β receptors but not homomeric 5-HT₃B receptors (Davies et al., 1999), it is proposed that nearly all functional 5-HT₃ receptors in the PNS and CNS of 5-HT₃A/– mice are hypersensitive to 5-HT and constitutively active. Constitutive activity of a cation-selective excitatory channel could lead to either functional desensitization of the receptor or neuron (as produced by capsaicin or c-fiber sensory afferents), or to persistent neuronal hyperexcitability followed by excitotoxic cell death. In support of the latter hypothesis, SCG neurons from 5-HT₃A/– mice had decreased levels of 5-HT mRNA, showed marked deficits in 5-HT elicited whole-cell currents, and the few neurons that did respond to 5-HT displayed significant increases in 5-HT potency. Although we have no direct evidence for constitutively
Table 3. Conscious open-filling cystometry parameters in 5-HT₃A wild-type and mutant mice

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (weeks)</th>
<th>Void pressure (mmHg)</th>
<th>Void interval (min)</th>
<th>Void volume (ml)</th>
<th>Overflow incontinence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>Male</td>
<td>8</td>
<td>73.3 ± 22.1</td>
<td>8.0 ± 2.6</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>vs/+</td>
<td>Male</td>
<td>8</td>
<td>c.d.</td>
<td>c.d.</td>
<td>c.d.</td>
</tr>
<tr>
<td>vs/vs</td>
<td>Male</td>
<td>8</td>
<td>c.d.</td>
<td>c.d.</td>
<td>c.d.</td>
</tr>
<tr>
<td>+/-</td>
<td>Female</td>
<td>8</td>
<td>42.7 ± 8.2</td>
<td>10.5 ± 3.4</td>
<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>vs/+</td>
<td>Female</td>
<td>8</td>
<td>31.0 ± 3.5</td>
<td>6.1 ± 1.1</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>vs/vs</td>
<td>Female</td>
<td>8</td>
<td>c.d.</td>
<td>c.d.</td>
<td>c.d.</td>
</tr>
<tr>
<td>+/-</td>
<td>Male</td>
<td>12</td>
<td>61.0 ± 8.1</td>
<td>5.4 ± 0.5</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>vs/+</td>
<td>Male</td>
<td>12</td>
<td>41.7 ± 6.8</td>
<td>7.2 ± 0.4</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>vs/vs</td>
<td>Male</td>
<td>12</td>
<td>c.d.</td>
<td>c.d.</td>
<td>c.d.</td>
</tr>
<tr>
<td>+/-</td>
<td>Female</td>
<td>12</td>
<td>50.7 ± 6.3</td>
<td>6.5 ± 1.4</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>vs/+</td>
<td>Female</td>
<td>12</td>
<td>39.7 ± 4.9</td>
<td>4.2 ± 0.5</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>vs/vs</td>
<td>Female</td>
<td>12</td>
<td>c.d.</td>
<td>c.d.</td>
<td>c.d.</td>
</tr>
</tbody>
</table>

c.d., Constant dribblers.

*For each group of mice, the number of overflow incontinent animals is shown against the total number of animals per group (in parentheses).

instability (Turner and Brading, 1997; Lluel et al., 1998; Charlton et al., 1999; Mills et al. 2000; Pandita et al., 2000). Denervation of the detrusor has been most widely associated with loss of postganglionic parasympathetic nerves, and it has been suggested that these areas of patchy denervation are responsible for both decreased nerve-evoked contractility and increased excitability contributing to bladder instability (Levin et al., 1995; Turner and Brading, 1997). Decreased innervation and loss of nerve-evoked bladder contractility, together with bladder hypertrophy and overflow incontinence, is consistent with an outlet obstruction phenotype in 5-HT₃A Clock mice. The current data would suggest that persistent activation of 5-HT₃A receptors, and excitotoxic neuronal cell loss, played a principal role in the development of denervation-induced changes in the urinary bladder of these mice.

The localization of 5-HT₃A receptors in the dorsal horn of the spinal cord and in peripheral sensory ganglia (TeCott et al., 1993; Johnson and Heinemann, 1995; Kia et al., 1995; Morales and Wang, 2002) suggests that excitotoxic cell death could affect sensory afferents innervating the urinary bladder of 5-HT₃A Clock mice. In the DRG, 5-HT₃A receptors are expressed by myelinated Aδ afferents and a small population of unmyelinated...
processing, the latter via activation of GABAergic inhibitory interneurons (Alhaider et al., 1991; Zeitz et al., 2002). Thus, the loss of 5-HT₃-expressing neurons in the CNS might indirectly influence afferent input to the urinary bladder.

5-HT receptors on parasympathetic efferent fibers also modulate detrusor smooth muscle contraction (Barra et al., 1996; Sellers et al., 2000), and evidence supports a role for 5-HT₃ receptors in the lower urinary tract. In the rabbit, for example, excitatory effects of 5-HT on the detrusor smooth muscle have been attributed to presynaptic 5-HT₃ receptors on parasympathetic efferents (Chen, 1990; Barra et al., 1996). Interestingly, in a rabbit model of BOO, an increased density of [³H]-5-HT-binding sites was observed in the detrusor smooth muscle (Khan et al., 1999), although the receptor subtype responsible for 5-HT binding was not characterized. Detrusor instability in rabbit BOO models is also associated with partial denervation and cholinergic supersensitivity (Speckman et al., 1987; Rohrmann et al., 1997). It is possible that in 5-HT₃A mutant mice, hypersensitive and constitutively active 5-HT₃A receptors on parasympathetic nerve fibers innervating the urinary bladder contributed to excitatory responses in the detrusor, with eventual neurotoxic cell death. 5-HT₃A mice did show a slight age-related loss of neurogenic bladder contractions and a decreased density of PGP 9.5 immunoreactive nerve fibers innervating the bladder. This latter observation is consistent with the findings in a recently described model of murine outlet obstruction, where bladder hypertrophy and overactivity were associated with decreased PGP 9.5, nitric oxide synthase, and vesicular ACh transporter immunoreactive nerve terminals in the detrusor smooth muscle (Pandita et al., 2000).

Based on our current data, it remains uncertain whether changes in the urethra contributed to the obstructive pathology seen in 5-HT₃A mice. Although no changes were observed in urethral biomechanical properties between 5-HT₃A and 5-HT₃A mice, we cannot rule out the possibility of alterations in physiological pathways not measured by this approach, such as the modulation of urethral smooth muscle relaxation by nitric oxide. Constitutive activation of 5-HT₃ receptors could also have caused obstruction in other outlet organs such as the bladder neck. Inflammation and hyperplasia of the prostatic urethra in male 5-HT₃ mice was also a probable contributor to bladder outlet obstruction, and this is consistent with the exacerbated disease process observed in male versus female mutant mice.

In summary, our findings suggest a role for 5-HT receptors in the development of an outlet obstructive pathology associated with bladder instability and overflow incontinence and provide the first report associating a hypersensitive gain of function mutation in the 5-HT₃ receptor with a neuro-urological pathology. The phenotype of this strain contrasts with that of 5-HT₃ receptors in null mice (Zeitz et al., 2002), which display reduced tissue injury-induced persistent nociception but no obvious urinary tract malfunction. These findings emphasize that complementary information can be obtained from gain of function and loss of function genetic manipulations (Lester et al., 2003). The knock-in approach for studying gain of function mutations has been described previously for nACh receptors, where point mutations in the M2 domain of the α7 (Orr-Urtreger et al., 2000; Broide et al., 2002) and α4 (Labarca et al., 2001) nACh receptor subunits resulted in hypersensitivity to both acetylcholine and nicotine in oocytes in vitro. Similar to 5-HT₃A mice, expression of hypersensitive α4 and α7 nACh receptors in vivo resulted in reduced levels of receptor subunit protein levels, neuronal cell death, and phenotypic behavioral changes (Labarca et al., 2001;
Broide et al. (2002). Interestingly, previous studies have also shown that knock-out mice lacking either the α3 subunit or both the β2 and β4 subunits of the nACh receptor had profoundly enlarged bladders with overflow incontinence, and developed urinary tract infections and bladder stones (Xu et al., 1999a; Xu et al., 1999b). Taken together, these data may help us to better understand the role of ligand-gated ion channels such as the 5-HT3 and nACh receptors in the neurophysiologic control of the bladder and the lower urinary tract.

References


