THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 2, pp. 930–937, January 9, 2009
© 2009 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Spermine Synthase Deficiency Leads to Deafness and a Profound Sensitivity to α-Difluoromethylornithine*◆

Received for publication, October 7, 2008, and in revised form, November 3, 2008 Published, JBC Papers in Press, November 10, 2008, DOI 10.1074/jbc.M807758200

Xiaojing Wang[‡], Snezana Levic[§], Michael Anne Gratton[¶], Karen Jo Doyle[§], Ebenezer N. Yamoah[§], and Anthony E. Pegg[‡1]

From the [‡]Department of Cellular and Molecular Physiology, Milton S. Hershey Medical Center, Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033, the [¶]Department of Otorhinolaryngology, University of Pennsylvania, Philadelphia, Pennsylvania 19014, and the [§]Center for Neuroscience, Program in Communication Sciences, University of California Davis, Davis, California 95618

Male gyro (Gy) mice, which have an X chromosomal deletion inactivating the *SpmS* and *Phex* genes, were found to be profoundly hearing impaired. This defect was due to alteration in polyamine content due to the absence of spermine synthase, the product of the *SpmS* gene. It was reversed by breeding the Gy strain with CAG/SpmS mice, a transgenic line that ubiquitously expresses spermine synthase under the control of a composite cytomegalovirus-IE enhancer/chicken β-actin promoter. There was an almost complete loss of the endocochlear potential in the Gy mice, which parallels the hearing deficiency, and this was also reversed by the production of spermine from the spermine synthase transgene. Gy mice showed a striking toxic response to treatment with the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO). Within 2–3 days of exposure to DFMO in the drinking water, the Gy mice suffered a catastrophic loss of motor function resulting in death within 5 days. This effect was due to an inability to maintain normal balance and was also prevented by the transgenic expression of spermine synthase. DFMO treatment of control mice or Gy-CAG/SpmS had no effect on balance. The loss of balance in Gy mice treated with DFMO was due to inhibition of polyamine synthesis because it was prevented by administration of putrescine. Our results are consistent with a critical role for polyamines in regulation of Kir channels that maintain the endocochlear potential and emphasize the importance of normal spermidine:spermine ratio in the hearing and balance functions of the inner ear.

Polyamines are essential for viability in mammals. Knockouts of the genes for ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, which are enzymes needed for the synthesis of putrescine, spermidine, and spermine, are lethal at early stages of embryonic development (1, 2). There is convincing evidence that the formation of hypusine in eIF5A, which requires spermidine as a precursor, is essential for eukaryotes (3). However, the function(s) of spermine is not so well established. Yeast mutants with inactivated spermine synthase grow at a normal rate (4). Mammalian cells in culture also grow normally in the presence of inhibitors of spermine synthase (5) or after inactivation of the spermine synthase gene (*SpmS*) (6–8). Inactivation of both of the genes that were originally described as encoding spermine synthases in plants leads to profound developmental defects (9–11), but recently it was discovered that one of these genes actually encodes a thermospermine synthase, and it appears that the lack of thermospermine may be responsible for these defects (12).

In contrast, spermine is clearly required for normal development in mammals. The rare human Snyder-Robinson syndrome is caused by mutations in *SpmS* located in the X chromosome that drastically reduces the amount of spermine synthase (13, 14). This leads to mental retardation, hypotonia, cerebellar circuitry dysfunction, facial asymmetry, thin habitus, osteoporosis, and kyphoscoliosis. Male mice, which have an X chromosomal deletion that includes *SpmS* and have no detectable spermine synthase activity, do survive but are only viable on the B6C3H background (15–17). This mouse strain having an X-linked dominant mutation was isolated from a female offspring of an irradiated mouse and was termed gyro (Gy)[2] based on a circling behavior pattern in affected males (18). Subsequent studies have shown that the Gy mice have a deletion of part of the X chromosome that inactivates both *Phex,* a gene that regulates phosphate metabolism, and *SpmS* (16, 19). The lack of *SpmS* causes a total absence of spermine (6, 7, 15, 16). Such Gy mice suffer from hypophosphatemia, have a greatly reduced size, sterility, and neurological abnormalities, and have a short life span (6, 16, 18). All of these changes except the hypophosphatemia are reversed when spermine synthase activity is restored (20).

The original characterization of Gy mice also reported preliminary indications that these mice had hearing defects lacking the Preyer reflex (21, 22). This is of particular interest in the context of polyamine metabolism because a drug, α-difluoromethylornithine (DFMO, Eflornithine), that targets ornithine decarboxylase has been shown to cause occasional hearing loss in some patients (23–26). Although DFMO was ineffective for cancer treatment, it is an extremely promising agent for cancer

* This work was supported, in whole or in part, by National Institutes of Health (USPHS) Grants R01 GM26290 (to A. E. P.) and DC007592 (to E. N. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
◆ This article was selected as a Paper of the Week.
[1] To whom correspondence should be addressed. Tel.: 717-531-8152; Fax: 717-531-5157; E-mail: aep1@psu.edu.

[2] The abbreviations used are: Gy, gyro; SPS, mice expressing a spermine synthase transgene; DFMO, α-difluoromethylornithine; DPOAE, distortion product otoacoustic emission; EP, endocochlear potential; MTA, 5′-methylthioadenosine; dcAdoMet, decarboxylated *S*-adenosylmethionine; dB, decibels; SPL, sound pressure level.

chemoprevention (27, 28). When combined with sulindac, DFMO treatment produced a substantial reduction in the recurrence of colorectal adenomas in a large clinical trial (27). DFMO is a major drug for the treatment of African sleeping sickness caused by *Trypanosoma brucei* (29, 30). It is also used as a topically applied cream for treatment of unwanted facial hair in women (31, 32). DFMO is generally well tolerated even at high doses, but reversible hearing loss has been reported in multiple clinical trials (25, 33), and a rarer irreversible defect has also been reported (34). These side effects are not observed at lower doses of DFMO (26, 27).

Ototoxicity has been demonstrated to occur in experimental animals treated with DFMO including rats (35), guinea pigs (36), gerbils (37), and mice (38). Using immunohistochemistry, a high level of ornithine decarboxylase was observed in the inner ear of the rat, with the highest in the organ of Corti and lateral wall followed by the cochlear nerve (39). Measurements of polyamines in the relevant structures are very difficult due to the small amount of tissue available, but as expected, DFMO treatment reduced polyamine levels and ornithine decarboxylase activity in the inner ear of the guinea pig (36). A plausible explanation for the importance of polyamines in auditory physiology is based on their well documented role as regulators of potassium channels (38). The inward rectification of Kir channels is caused by blockage of the outward current by polyamines (40–42). Studies of the cloned mouse cochlear lateral wall-specific Kir4.1 channel showed that inward rectification was reduced and that there was a marked reduction in endocochlear potential (EP). It was proposed that DFMO treatment increases the outward Kir4.1 current, resulting in a drop in EP (38).

In the experiments reported here, we have studied in more detail the role of polyamines in auditory physiology using Gy mice and crosses of these mice with transgenic CAG/SpmS mice (43). These mice express spermine synthase under the control of a composite cytomegalovirus-IE enhancer/chicken β-actin promoter, which was designed to provide ubiquitous expression (44–46). Assays of the spermine synthase activity in CAG/SpmS line 8 confirmed that there was a high level of expression of the transgene in many different organs and that this level was maintained for at least 1 year (43). Our studies confirm that Gy mice are totally deaf and that this condition is reversed by the expression of the *SpmS* gene. These changes are due to a virtually complete loss of the EP in the Gy mice. We have also examined the effect of DFMO on the Gy mice. Unexpectedly, it was found that these mice show a rapid and profound toxicity to this drug, leading to death within a few days. Within 5 days of exposure to DFMO in the drinking water, the DFMO-treated mice suffered a catastrophic loss of balance due to inner ear effects. This toxicity was also prevented by the transgenic expression of spermine synthase in the Gy background.

EXPERIMENTAL PROCEDURES

Materials

All chemicals, unless noted, were purchased from Sigma. Oligodeoxyribonucleotides used as primers were synthesized in the Macromolecular Core Facility, Pennsylvania State University College of Medicine.

Polyamine

Polyamine content was determined by high pressure liquid chromatography using an ion-pair reverse phase high performance liquid chromatography separation method with post-column derivatization using *o*-phthalaldehyde (47).

Mice

Heterozygous Gy mice were a kind gift from Dr. R. A. Meyer, Jr., of the Department of Orthopedic Surgery, Carolinas Medical Center, Charlotte, NC. Further animals were purchased from Jackson Laboratory (Bar Harbor, ME). XGy females were bred with B6C3H males from The Jackson Laboratory. Female offspring were retained, and the heterozygous females required for breeding were distinguished from homozygous wild type females by phosphate analyses of blood plasma (18, 43). The Gy males resulting from the breeding of these mice were identified by PCR using genomic DNA isolated from the tails as described previously (43).

The CAG/SpmS mice were generated by DNA microinjection of fertilized B6D2F2 oocytes using a 3.5-kb fragment released by SalI and BamHI digestion from plasmid pCAG-hSpmSyn, which was constructed by replacing the insert encoding green fluorescent protein in plasmid pCX-EGFP with the human spermine synthase cDNA (43). Genomic DNA was isolated from the tails and subjected to PCR analysis to identify mice bearing the transgene. For identification of the CAG/SpmS, the 5′ sense primer used was 5′-TTCGGCTTCTGGCGTGTGAC-3′, which corresponds to a sequence in the actin promoter region, and the 3′ antisense primer was 5′-CCAGTACTGTCCTGACTC-3′, which corresponds to nucleotides 300–317 in the spermine synthase coding sequence. A 440-bp fragment was produced from the transgene.

Phosphate Assays

Plasma level of phosphate was measured by a colorimetric assay. The blood from a retro-orbital bleed was collected in heparinized microcentrifuge tubes and spun at 14,000 rpm (15,000 $\times$ *g*) for 15 min, and then plasma was prepared for analysis. 1.35 ml of 10% trichloroacetic acid was added to the microcentrifuge tubes along with 20 μl of plasma, and then samples were vortexed and spun at 14,000 rpm (15,000$\times$ *g*) for 10 min. 1 ml of supernatant was placed into a clean glass tube, diluted with 2 ml of a solution containing 2% ascorbic acid, 0.5% ammonium molybdate, and 1.2 N H_2SO_4, and vortexed. Samples were then incubated at 37 °C for 90 min and cooled to room temperature for 10 min, and absorbance was measured on a spectrophotometer at 820 nm.

Assessment of Auditory and Vestibular Functions

Gross Assessment—We assessed the ear twitch response of mice with a hand clap (Preyer reflex) to grossly assess the hearing functions. To obtain a gross assessment of the vestibular function of mice, we performed a balance test (48, 49). Balance was tested by placing mice on a soft fabric-covered horizontal cylinder (~7 cm in diameter) and positioned 10 cm above a foam pad. The cylinder was connected to a variable speed motor that runs from 0 to 15 rpm. The ability of each animal to balance on both the stationary cylinder and the rotating cylinder was scored as balanced index (49).

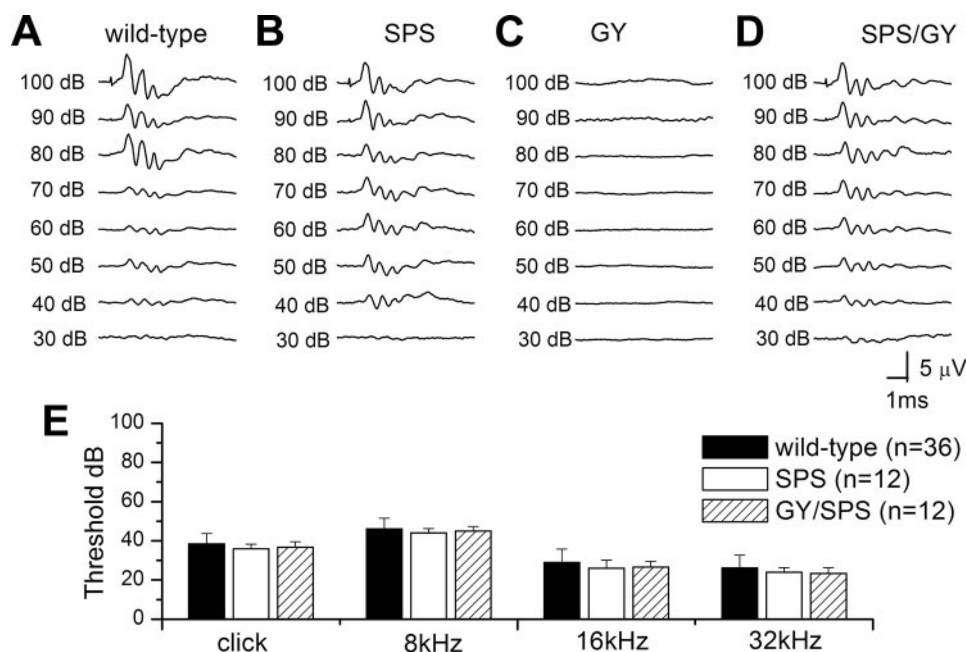

FIGURE 1. **Hearing threshold in control, Gy, SPS, and SPS/Gy mice.** The SPL in dB of broadband clicks (0.1 ms) delivered to the ear are indicated on the *left side* of the traces. Representative normal broadband click responses from control (*A*), SPS (*B*), Gy (*C*), and SPS/Gy (*D*) mice are shown. The auditory brainstem response responses over the range of 30–100 dB for representative mice from each group are shown. No response was observed from any of the 10 Gy mice examined. *E*, auditory brainstem response thresholds for wild type ($n = 36$), SPS ($n = 12$), and SPS/Gy ($n = 12$) mice in response to broadband clicks and 3-ms pure tones of 8, 16, and 32 kHz ($p = 0.5$ at 8 kHz, $p = 0.6$ at 16 kHz, and $p = 0.4$ at 32 kHz). The data are means ± S.D. Gy mice showed no detectable response to any stimulus.

Endocochlear Potential Measurement—The EP was measured as described briefly (38). The mice were anesthetized with 20% urethane (0.01 ml/g). A tracheal cannula was inserted, and the bulla was opened using a retroauricular approach. A small hole was made in the bony wall of the cochlea over the basal turn. A glass micropipette electrode filled with 150 mM KCl was advanced through the hole while secured to a hand-controlled micromanipulator. The electrode was connected to a high impedance direct current amplifier. The reference electrode (silver/silver chloride pellet) was placed under the dorsal skin. The recording electrode passed through the spiral ligament of the lateral wall into the scala media. The potential difference (mV) between the scala media and the reference electrode was recorded.

Light Microscopic Cochlear Morphology—Animals were anesthetized (Avertin, (2,2,2-tribromethanol), 300 μg/g of body weight, intraperitoneally) and transcardially perfused with phosphate-buffered saline (5 ml, 23 °C) followed by a solution of 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (10 ml, pH 7.4, 23 °C). The temporal bones were removed and opened to expose the otic capsule. The stapes was removed, and a perforation was made in the round window, following which fixative was perfused through the oval window. The bulla was then immersed in fixative (12 h, 4 °C), rinsed with phosphate buffer, postfixed (1% OsO_4 in 0.1 M phosphate buffer, 30 min), and decalcified (80 ml, 120 mM, disodium EDTA, 23 °C). The cochleae were dehydrated through a graded series of ethanols and propylene oxide prior to infiltration and were embedded in plastic resin (EmBed 812, Electron Microscopy Sciences, Fort Washington, PA). After polymerization (12 h, 58 °C), the cochlea was bisected in the mid-modiolar plane. The half-cochlea were re-embedded and completely polymerized (18 h, 58 °C). Semi-thick sections (1 μm) in the mid-modiolar were cut and stained with toluidine blue for light microscopic examination. Images were captured using an Olympus BH-2 microscope fitted with a SPOT RT-KE CCD camera and image analysis software (Diagnostic Instruments, Sterling Heights, MI). Final figures were assembled using Adobe PhotoShop and Illustrator software (Adobe Systems, San Jose, CA).

Auditory Brainstem Responses—Mice were anesthetized with Avertin, and auditory brainstem response measurements were recorded as described previously (50). Briefly, a ground needle electrode and recording needle were placed subcutaneously in the scalp, and then a calibrated electrostatic speaker coupled to a hollow ear bar was placed inside the pinna. Broadband clicks and pure tones (8, 16, and 32 kHz) were presented in the ear of the animal in 10-dB increments, starting from 0 dB of SPL and ending at 100 dB of SPL. The auditory brainstem response sweeps were computer-averaged (time-locked with onset of 128–1024 stimuli, at 20/s) out of the continuous electroencephalographic activity. The threshold of hearing was determined as the lowest intensity of sound required to elicit a characteristic waveform.

Distortion Product Otoacoustic Emissions (DPOAE)—Mice were anesthetized with ketamine (95 mg/kg) and xylazine (4 mg/kg). The f_1 and f_2 primary tones, which were generated by a two-channel frequency synthesizer (Hewlett Packard 3326A), were presented over two tweeters (Realistic) and delivered through a small soft rubber probe tip. Ear canal sound pressure was measured with a commercial acoustic probe (Etymotic Research 10B$^+$). The ear canal sound pressure was sampled and synchronously averaged ($n = 8$) by a digital signal processor for frequencies <20.1 kHz and by a dynamic signal analyzer (Hewlett Packard 3561A) for frequencies >20.1 kHz. Distortion products were collected over a range of geometric mean frequencies between 5.6 and 48.5 kHz ($f_2 = 6.3$–54.2 kHz) in 0.5-octave intervals at stimulus levels of $L_1 = L_2 = 65$ dB of SPL with $f_2/f_1 = 1.25$.

RESULTS

XGy carrier females were bred with transgenic male CAG/SpmS mice from CAG/SpmS line 8 (43), and the male offspring (control, Gy, SPS, and SPS/Gy) were tested for hearing and cochlear function. As shown in Fig. 1, no response was seen in the Gy mice at any frequency tested. The hearing defect was completely reversed by the transgenic expression of spermine

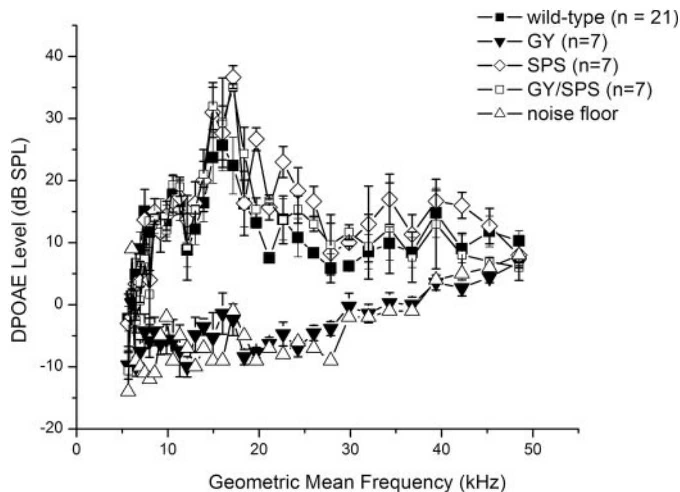

FIGURE 2. **DPOAE testing of control, Gy, SPS, and SPS/Gy mice.** Mean distortion products for 7–8-week-old control (wild type, $n = 21$), Gy ($n = 7$), SPS ($n = 7$), and SPS/Gy mice were tested measuring the levels of the 2 $f_1 - f_2$ DPOAE over a geometric-mean frequency range from 5.6 to 48.5 kHz, using an f_2/f_1 of 1.25 and primary tone stimuli at $L_1 = L_2 = 65$ SPL. Clearly, Gy mice yielded no significant DPOAEs at low-to-high frequencies (5.6–45 kHz) when compared with controls, SPS, and SPS/Gy mice.

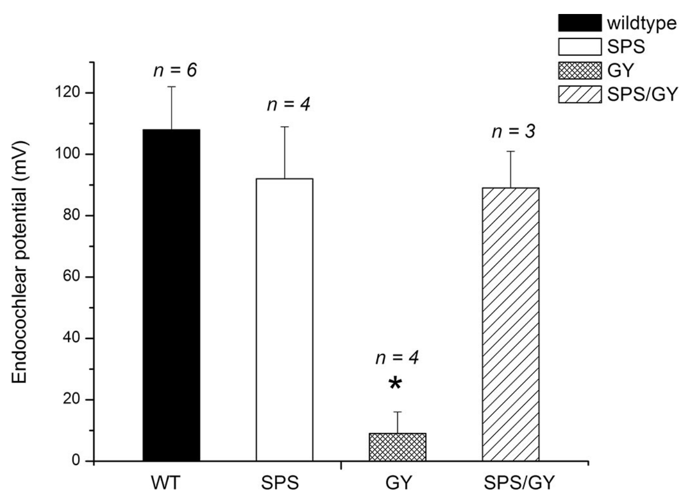

FIGURE 3. **EP in control, Gy, SPS, and SPS/Gy mice.** Gy mice had a significant reduction in EP at the basal turn of the cochlea, control/wild type (*WT*) = 108 ± 14 mV ($n = 6$); SPS = 92 ± 17 mV ($n = 4$); Gy = 9 ± 7 mV ($n = 4$) and SPS/Gy = 89 ± 12 mV. EP data obtained from the apical turn were wild type = 101 ± 8 mV ($n = 3$); SPS = 91 ± 13 ($n = 3$); Gy = 6 ± 5 ($n = 3$), and SPS/Gy = 85 ± 14 mV ($n = 3$).

synthase. The control, SPS, and SPS/Gy groups showed identical responses (Fig. 1). Similarly, DPOAE testing showed no difference between the response of Gy mice and the noise floor, whereas the SPS and SPS/Gy groups showed a similar response to the controls (Fig. 2). These results confirm and extend the preliminary abstracts, stating that there was an absence of the Preyer reflex in Gy mice (21, 22).

There was an almost complete loss of the EP in the Gy mice, which parallels the hearing deficiency (Fig. 3). The loss of EP was also abolished by the provision of transgenic spermine synthase in the SPS/Gy mice.

The cochlear tissues of the SPS and SPS/Gy mice appear normal with the exception of an increased width of the stria vascularis (Fig. 4, *A–C*). Examination of the stria at higher magnification indicated normal basal cells as well as marginal cell

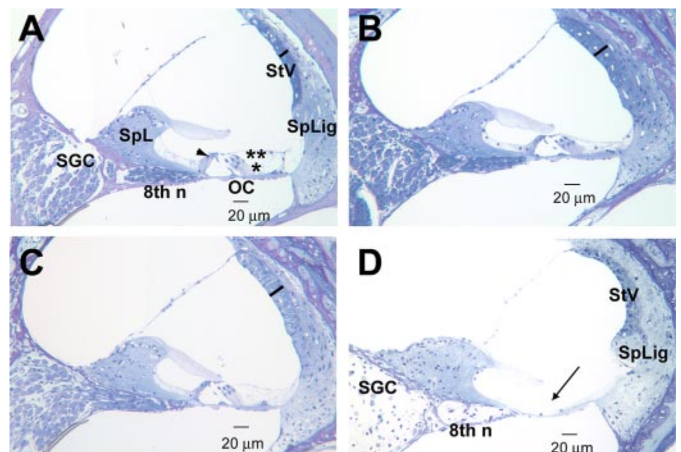

FIGURE 4. **Histology of Scala media of the lower apical turn of the cochlea in control (*A*), SPS (*B*), SPS/Gy (*C*), and Gy (*D*) mice.** *A*, a cross-section of the scala media from a control cochlea shows a normal complement of spiral ganglion cells (*SGC*), and organ of Corti (*OC*) with inner hair cell (*arrowhead*) and three outer hair cells (*double asterisk*), each supported by a Deiters cell (*asterisk*). The lateral wall consists of the stria vascularis (*StV*) of expected density and thickness (*black bar*) and fibrocytes of the spiral ligament (*SpLig*). *B*, the cross-section of the SPS cochlea appears normal. Increased thickness (*black bar*) is noted in the stria vascularis. *C*, increased strial width (*black bar*) is the only aberrant feature noted in the cochlea of the SPS/Gy mouse. *D*, the cochlea of the Gy mouse lacks eighth nerve fibers (*8th n.*) and spiral ganglion cells. The hair cells and supporting cells of the organ of Corti have been replaced by a layer of squamous epithelia cells (*arrow*). The fibrocytes of the spiral ligament (*SpLig*), and the stria vascularis (*StV*) appear grossly normal. *SpL*, spiral limbus.

bodies, suggesting that the increased width was due to the increased area of the intermediate cells and the interdigitations of the intermediate cells with the basolateral processes of the marginal cells. In the SPS/Gy cochlea (Fig. 4*C*), some intermediate cell nuclei are located in unusually close apposition to the basal cells. In one SPS/Gy mouse, a layer of squamous epithelial cells covered the basilar membrane instead of the organ or Corti in the lower basal turn, and reduced eighth nerve fibers were noted. The Gy mice lack outer hair cells, and inner hair cells are observed only in the basal turn (Fig. 4*D*). Also absent, or sparsely present, are spiral ganglion cells and eighth nerve fibers. The cellular architecture in the lateral wall varies. In some turns, the spiral ligament appears normal, and the stria has a normal density and thickness. In other turns, the stria has atrophied, consisting of a layer of squamous epithelial cells overlaying remnants of capillaries and basal cells. In these turns, the spiral ligament often shows that extracellular matrix has replaced many of the fibrocytes. A consistent feature throughout the Gy lateral wall is the presence of lipid (Fig. 4*D*). The lipid appears as uniformly dense, membrane-bound, and usually round intracellular inclusions of varying sizes. These droplets can be found in all cell types of the stria and spiral ligament as well as occasionally in cells of the organ of Corti and spiral limbus. Degenerating cochlear cells present in the endolymph can also contain lipid inclusions.

Attempts were made to restore spermine by direct administration either in diet or by injection. It is quite surprising that previous studies have found that tissues from Gy mice contain no spermine (6, 7, 15, 16, 43) because normal mouse diets contain significant amounts of spermine. It appears possible that the presence of even higher amounts of spermidine in the food

(which is likely increased further by spermidine from microbial flora) prevented the uptake of dietary spermine from the gut. We therefore formulated a synthetic diet lacking polyamines, in which egg white protein was the major protein source and dextrose and corn oil were sources of carbohydrate and lipid, and supplemented it with large amounts of spermine (1.71 g/kg) and no spermidine. However, even after feeding this diet for 3 weeks, there was no significant amount of spermine in any of the tissues (heart, liver, kidney, brain, intestine) examined. We then tested the administration of spermine by intraperitoneal injection of a solution in 0.9% NaCl. Due to the known toxicity of such injections (51), the maximum daily dose that could be administered in this way was only 7 mg/kg. After 21 days of treatment (daily injections for 3 days followed by injections every other day), the mice were killed 8 h after the last injection. Significant spermine uptake was seen in some tissues such as kidney, liver, and heart but not in the brain. Spermine was not restored to control levels, being only 70% of control in kidney, 20% in liver, and 22% in heart, and the spermidine:spermine ratio was still much higher than that seen in normal mice. Attempts to administer spermine via the implantation of slow release pellets containing 0.84 mg of spermine tetrahydrochloride were even less successful in restoring spermine levels with 5.9% in kidney, 5.6% in liver, 7.3% in heart, and <0.1% in brain after 21 days.

One explanation for the relatively poor tissue uptake of spermine would be that the very high level of spermidine in Gy mice (6, 7, 15, 16) represses the polyamine transporter, which is known to be greatly reduced in activity by a high cellular polyamine content (52–54). Conversely the activity of this transporter is increased significantly by treatment with DFMO, which blocks the biosynthetic pathway at the ornithine decarboxylase step. We therefore decided to test the ability of treatment with DFMO, which was administered in the drinking water, to increase spermine uptake. Mice were placed on the spermine-enriched diet and given DFMO. The dose of DFMO that was used (1% in the drinking water) was based on numerous studies in the literature showing that this dose is well tolerated in rodents (55, 56), and this was confirmed in the control group of mice. However, the Gy mice suffered a severe toxic effect in response to the DFMO, and all died within 5 days. Observation of these mice showed that they rapidly developed an inability to maintain normal posture. Investigation of their balance index (Fig. 5) indicated that they were totally unable to maintain normal balance. This effect was prevented by the transgenic expression of spermine synthase. The SPS and SPS/Gy groups showed a normal balance index similar to that of the control mice (Fig. 5), and this index was not affected by DFMO treatment.

The striking toxic effect of DFMO on the Gy mice was also obvious in the body weight of the treated mice (Table 1 and Fig. 6). Even after DFMO treatment, control mice continued to gain weight to the same extent as the non-treated controls, but DFMO caused a significant net weight loss in the Gy mice. This toxicity was only slightly ameliorated by provision of spermine, but as described above, such injection of spermine only slightly raised tissue spermine levels. Exposure to L-ornithine in the drinking water had no effect on the Gy mice (Fig. 6) or control

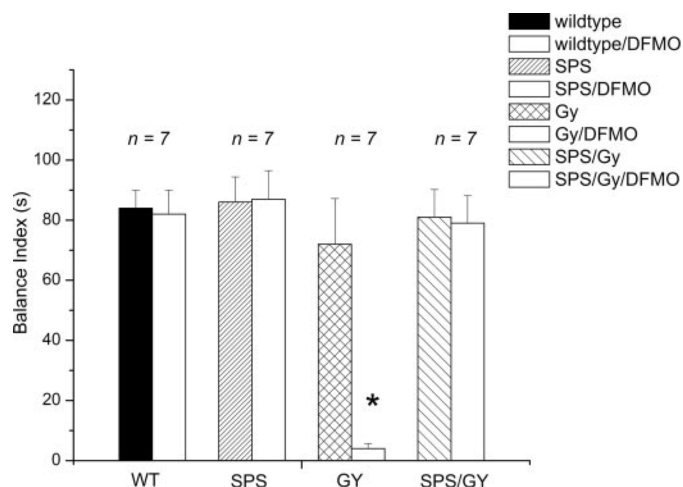

FIGURE 5. **Balance index in control, Gy, SPS, and GySPS mice before and after 1.5-day treatment with DFMO.** The times taken to remain on a rotating cylinder (5 rpm) were defined as the balance index as described (49) and are illustrated in the histogram. Data were obtained from DFMO pre- and post-treated mice. Seven animals from each genotype were tested. The mean of 10 trials for each animal is reported. The *asterisk* indicates significant differences ($p < 0.01$) between pre- and post-treated animals. To eliminate fatigue factor, each trial was separated by a 10-min rest period. No reward was given to the animals during or after the test. *Unshaded bars* show results after DFMO treatment. *WT*, wild type.

TABLE 1
Body weight of Gy and control mice treated with DFMO

Group	Mean of % of weight gained in 4 days	
	Gy	Control
No treatment	17.5 ± 1.6	15.6 ± 2.2
DFMO	−4.7 ± 2.9	18.7 ± 2.3
DFMO + Spermine[a]	0.0 ± 2.0	20.3 ± 1.9
Spermine[a]	15.1 ± 2.4	11.5 ± 3.0

[a] Spermine was administered by daily intraperitoneal injections of 7 mg/kg.

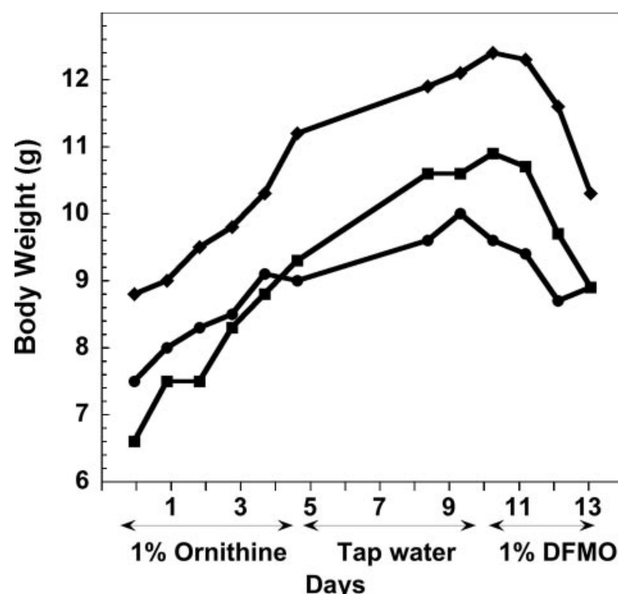

FIGURE 6. **Effect of L-ornithine and DFMO on body weight of Gy mice.** Three Gy mice (4–6 weeks in age) were exposed to L-ornithine by placing 1% in the drinking water for 5 days, and the drinking water was then replaced by water with no additions for 5 days and then changed to water containing 1% DFMO. The body weight of each mouse was measured as shown. No behavioral changes were seen until the DFMO administration, when all mice developed the inability to maintain posture described under "Results."

mice (not shown), but when the ornithine was replaced by DFMO, the toxicity was observed (Fig. 6).

The toxicity of DFMO in Gy mice described above was observed in mice that were 3–4 weeks old, but a similar effect was seen in mice that were 2–4 months old. These mice also showed the inability to gain weight and a loss of balance described above when exposed to DFMO. Administration of putrescine (500 mg/kg) by intraperitoneal injection of a solution in 0.9% NaCl daily for 4 days completely prevented this effect (results not shown), and the DFMO- and putrescine-treated Gy mice gained 3.9% body weight in 3 days when compared with a loss of 13% in the Gy mice given DFMO alone ($n = 4$/group).

Because Gy mice have an X chromosome deletion that extends into the *Phex* gene (whose inactivation causes hypophosphatemia (16, 19)), it was important to rule out the involvement of the Phex gene product in the response to DFMO. We therefore tested Hyp mice that also have a deletion of the X chromosome, which inactivates the *Phex* gene but does not extend into *SpmS* (16, 57, 58). The Hyp mice showed no toxic effects on exposure to DFMO (results not shown), and DFMO-treated Hyp mice gained weight at the same rate as the untreated controls.

DISCUSSION

Due to the extremely limited amount of tissue available, it is not possible to measure the polyamine content in the inner ear in the mice used in these experiments. Other studies with a wide variety of tissues have confirmed that the deletion of the *SpmS* gene in Gy mice leads to a loss of spermine and an increase in spermidine in all cells examined (6, 7, 15, 16). These changes in polyamine content are reversed by the spermine synthase transgene in SPS/Gy mice (43). Therefore, it is very likely that the loss of hearing in the Gy mice is caused by the alterations in polyamines, although whether it is the loss of spermine or the increase in spermidine (or both) that is responsible is not clear at present.

The very large reduction in EP in the Gy mice (Fig. 3) accounts for the loss in hearing, and this alteration is fully reversed to control levels in the SPS/Gy mice. A plausible explanation for the importance of polyamines in maintaining the EP is provided by studies showing that Kir channels are regulated by polyamines. Both spermidine and spermine can bring about the strong inward rectification of such channels, but spermine is more potent (42, 59, 60). The importance of Kir channels in maintaining EP was established by studies of the cloned mouse cochlear lateral wall-specific Kir4.1 channel (38). A high level of polyamines, particularly spermidine, was reported to occur in the rat cochlea (35), and spermidine levels were reduced by DFMO in the guinea pig cochlea (36).

Our results are the first to demonstrate directly the critical importance of normal spermine levels in maintaining EP and hearing, but it is noteworthy that an alteration in chromosome 9 that disrupts the *MTAP* gene has recently been reported as a source of heritable deafness (61). This gene encodes 5′-methylthioadenosine (MTA) phosphorylase, which degrades the MTA formed in polyamine synthesis (62). MTA is a potent inhibitor of spermine synthase (63, 64), and the loss of MTA phosphorylase activity would be expected to reduce spermine levels. Very potent inhibitors of MTA phosphorylase are currently under development as antitumor agents (65), and their possible ototoxicity should be evaluated.

The stria vascularis is a region of the lateral wall of the cochlear duct that is the powerhouse for the generation of the EP and for the production of high K^+ endolymph (~150 mM). The stria vascularis consists of marginal cells, intermediate cells, basal cells, and capillaries. In the stria vascularis of control, SPS, and SPS/Gy mice, marginal cells, which normally secrete K^+ and form a continuous sheet in contact with the endolymph, are columnar and have elaborate extensions of the basal region of the cell that results in an extensive plasmalemma surface area. The stria vascularis of the Gy mice also looked grossly normal. Although the cochlea and auditory nerve fibers of control, SPS, and SPS/Gy appear normal, the cochlea of the Gy mice lacked auditory nerve fibers and spiral ganglia neurons. Also prominent was the profound loss of hair cells and replacement of the organ of Corti by a layer of squamous epithelia cells. Loss of hair cells may ensue from significant reduction in the magnitude of the EP as seen in the Gy mice. Previous studies have demonstrated that loss of hair cells appears to be a hallmark of several animal models with similar EP phenotype (46). Presumably, the activity of hair cells imposed by the EP is necessary for their survival. Because activity of hair cells results in release of neurotrophic factors, which play an instructive role in the survival of spiral ganglia neurons, subsequent degeneration of the auditory neurons is expected in the Gy mice model.

It is also noteworthy that mice lacking the basolateral Na-K-2Cl cotransporter (NKCC1) are deaf and have difficulty maintaining their balance (50). This suggests that transepithelial K^+ movement is involved in generation of the EP. There are several other similarities in the phenotype of Gy and NKCC1 knock-out mice. Both show hyperactivity and circling behavior and are sterile due to lack of sperm development (6, 16, 43, 66, 67). NKCC1 knock-out mice have also been reported to show reduced weight gain and a tendency to sudden death, but these characteristics are more marked in Gy mice.

Many studies involving exposure of experimental animals or humans to DFMO have shown that DFMO is remarkably non-toxic with the most serious side effects seen in clinical trials involving hearing loss (Refs. 25–27, 33, and 34 and references therein). This effect is usually but not always rapidly reversible on discontinuing the drug. Many experimental studies with rodents have shown that exposure to DFMO at 1–2% in the drinking water for weeks has no serious effects on growth and development. Our studies, however, show that in the Gy mice, there was a major toxic effect of DFMO, leading to a profound weight loss and death within a few days. This toxicity appears to be centered on the inner ear because there was a profound loss of balance, preventing normal feeding and drinking. This effect was specific to the Gy mice and is clearly not related solely to their loss of the *Phex* gene product (which affects phosphate metabolism) because (*a*) it was not seen in Hyp mice, which also have a deletion of *Phex* but not of *SpmS,* and (*b*) it was reversed by the transgenic expression of spermine synthase. It is possible that the combined loss of spermine and Phex contributes to the effect. The toxicity is very likely to be a consequence of the

prevention of putrescine synthesis by DFMO because (*a*) it was not seen when ornithine was substituted for DFMO, and (*b*) it was reversed by co-administration of putrescine. Although we were unable to achieve more than a slight reduction in the toxicity by treatment with spermine, this treatment was not sufficient to fully restore the polyamine levels in the Gy mice.

In control animals and cells, DFMO treatment normally causes a rapid reduction of putrescine and spermidine without greatly affecting spermine (68, 69). In the guinea pig cochlea, there was a complete loss of putrescine and a small reduction in both spermidine and spermine after 4–8 weeks of treatment with DFMO concentrations of 2–4 g/kg/day, which caused a significant hearing loss (36). Although we could not measure these levels in the inner ear of the Gy mice due to the small amount of tissue available, it is likely that putrescine and spermidine are reduced and that, in the absence of spermine, this leads to a catastrophic failure of the balance mechanism.

Although the most likely explanation of our results is that they are related to changes in polyamine content, it should be noted that changes in polyamine metabolism may also affect the cellular pool of *S*-adenosylmethionine and its decarboxylated derivative (dcAdoMet), which is the substrate for aminopropyltransferases. Previous studies with DFMO have shown that both in isolated cells and in treated mice, there is a substantial rise in dcAdoMet content (70–72) due to the reduction in putrescine and spermidine needed for aminopropyl-transferase reactions. Similarly, there is an increase in dcAdoMet in tissues of Gy mice.[3] It is therefore possible that changes in the *S*-adenosylmethionine pool also contribute to the pathophysiology described in our studies.

REFERENCES

1. Pendeville, H., Carpino, N., Marine, J. C., Takahashi, Y., Muller, M., Martial, J. A., and Cleveland, J. L. (2001) *Mol. Cell. Biol.* **21,** 6459–6558
2. Nishimura, K., Nakatsu, F., Kashiwagi, K., Ohno, H., Saito, H., Saito, T., and Igarashi, K. (2002) *Genes Cells* **7,** 41–47
3. Park, M. H. (2006) *J. Biochem.* (*Tokyo*) **139,** 161–169
4. Hamasaki-Katagiri, N., Katagiri, Y., Tabor, C. W., and Tabor, H. (1998) *Gene* (*Amst.*) **210,** 195–210
5. Pegg, A. E., and Coward, J. K. (1985) *Biochem. Biophys. Res. Commun.* **133,** 82–89
6. Mackintosh, C. A., and Pegg, A. E. (2000) *Biochem. J.* **351,** 439–447
7. Nilsson, J., Gritli-Linde, A., and Heby, O. (2000) *Biochem. J.* **352,** 381–387
8. Korhonen, V.-P., Niranen, K., Halmekyto, M., Pietilä, M., Diegelman, P., Parkkinen, J. J., Eloranta, T., Porter, C. W., Alhonen, L., and Jänne, J. (2001) *Mol. Pharmacol.* **59,** 231–238
9. Imai, A., Akiyama, T., Kato, T., Sato, S., Tabata, S., Yamamoto, K. T., and Takahashi, T. (2004) *FEBS Lett.* **556,** 148–152
10. Clay, N. K., and Nelson, T. (2005) *Plant Physiol.* **138,** 767–777
11. Yamaguchi, K., Takahashi, Y., Berberich, T., Imai, A., Takahashi, T., Michael, A. J., and Kusano, T. (2007) *Biochem. Biophys. Res. Commun.* **352,** 486–490
12. Knott, J. M., Romer, P., and Sumper, M. (2007) *FEBS Lett.* **581,** 3081–3086
13. Cason, A. L., Ikeguchi, Y., Skinner, C., Wood, T. C., Lubs, H. A., Martinez, F., Simensen, R. J., Stevenson, R. E., Pegg, A. E., and Schwartz, C. E. (2003) *Eur. J. Hum. Genet.* **11,** 937–944
14. de Alencastro, G., McCloskey, D. E., Kliemann, S. E., Maranduba, C. M., Pegg, A. E., Wang, X., Bertola, D. R., Schwartz, C. E., Passos-Bueno, M. R., and Sertié, A. L. (2008) *J. Med. Genet.* **45,** 539–543
15. Lorenz, B., Francis, F., Gempel, J., Böddrich, A. J. M., Schmahl, W., and Schmidt, J. (1998) *Hum. Mol. Genet.* **7,** 541–547
16. Meyer, R. A., Jr., Henley, C. M., Meyer, M. H., Morgan, P. L., McDonald, A. G., Mills, C., and Price, D. K. (1998) *Genomics* **48,** 289–295
17. Meyer, R. A., Jr., Meyer, M. H., Gray, R. W., and Bruns, M. E. (1995) *J. Orthop. Res.* **13,** 30–40
18. Lyon, M. F., Scriver, C. R., Baker, L. R., Tenenhouse, H. S., Kronick, J., and Mandla, S. (1986) *Proc. Natl. Acad. Sci. U. S. A.*, 4899–4903
19. Grieff, M., Whyte, M. P., Thakker, R. V., and Mazzarella, R. (1997) *Genomics* **44,** 227–231
20. Wang, X., Ikeguchi, Y., McCloskey, D. E., Nelson, P., and Pegg, A. E. (2004) *J. Biol. Chem.* **49,** 51370–51375
21. Barkway, C., Glenn, N., Harvey, D., Moorjani, P. A., Palmer, A. R., Stabler, S., and Steel, K. P. (1988) *Hereditary Deafness Newsletter* **1,** 20–21
22. Barkway, C., Glenn, N., Moorjani, P. A., Palmer, A. R., Stabler, S., and Steel, K. P. (1989) *Hereditary Deafness Newsletter* **3,** 20–21
23. Horn, Y., Schechter, P. J., and Marton, L. J. (1987) *Eur. J. Cancer Clin. Oncol.* **23,** 1103–1107
24. Pasic, T. R., Heisey, D., and Love, R. R. (1998) *Arch. Otolaryngol. Head Neck Surg.* **123,** 1281–1286
25. Meyskens, F. L., Jr., and Gerner, E. W. (1999) *Clin. Cancer Res.* **5,** 945–951
26. Doyle, K. J., McLaren, C. E., Shanks, J. E., Galus, C. M., and Meyskens, F. L. (2001) *Arch. Otolaryngol. Head Neck Surg.* **127,** 553–558
27. Meyskens, F. L., Jr., McLaren, C. E., Pelot, D., Fujikawa-Brooks, S., Carpenter, P. M., Hawk, E., Kelloff, G., Lawson, M. J., Kidao, J., McCracken, J., Albers, C. G., Ahnen, D. J., Turgeon, D. K., Goldschmid, S., Lance, P., Hagedorn, C. H., Gillen, D. L., and Gerner, E. W. (2008) *Cancer Prev. Res.* 9–11
28. Simoneau, A. R., Gerner, E. W., Nagle, R., Ziogas, A., Fujikawa-Brooks, S., Yerushalmi, H., Ahlering, T. E., Lieberman, R., McLaren, C. E., Anton-Culver, H., and Meyskens, F. L., Jr. (2008) *Cancer Epidemiol. Biomark. Prev.* **17,** 292–299
29. Priotto, G., Pinoges, L., Fursa, I. B., Burke, B., Nicolay, N., Grillet, G., Hewison, C., and Balasegaram, M. (2008) *BMJ* **336,** 705–708
30. Barrett, M. P., Boykin, D. W., Brun, R., and Tidwell, R. R. (2007) *Br. J. Pharmacol.* **152,** 1155–1171
31. Hamzavi, I., Tan, E., Shapiro, J., and Lui, H. (2007) *J. Am. Acad. Dermatol.* **57,** 54–59
32. Hoffmann, R. (2008) *Eur. J. Dermatol.* **18,** 65–70
33. Abeloff, M. D., Rosen, S. T., Luk, G. D., Baylin, S. B., Zeltzman, M., and Sjoerdsma, A. (1986) *Cancer Treat. Rep.* **70,** 843–945
34. Lao, C. D., Backoff, P., Shotland, L. I., McCarty, D., Eaton, T., Ondrey, F. G., Viner, J. L., Spechler, S. J., Hawk, E. T., and Brenner, D. E. (2004) *Cancer Epidemiol. Biomark. Prev.* **13,** 1250–1252
35. Brock, M., and Henley, C. M. (1994) *Hear. Res.* **72,** 37–43
36. Marks, S. C., Mattox, D. E., and Casero, R. A. (1991) *Hear. Res.* **53,** 230–236
37. Smith, M. C., Tinling, S., and Doyle, K. J. (2004) *Laryngoscope* **114,** 1113–1117
38. Nie, L., Feng, W., Diaz, R., Gratton, M. A., Doyle, K. J., and Yamoah, E. N. (2005) *J. Biol. Chem.* **280,** 15097–15102
39. Henley, C. M., Salzer, T. A., Coker, N. J., Smith, G., and Haddox, M. K. (1995) *Hear. Res.* **84,** 99–111
40. Lopatin, A. N., N., M. E., and Nichols, C. G. (1994) *Nature* **372,** 366–369
41. John, S. A., Xie, L. H., and Weiss, J. N. (2004) *J. Gen. Physiol.* **123,** 623–625
42. Kurata, H. T., Marton, L. J., and Nichols, C. G. (2006) *J. Gen. Physiol.* **127,** 467–480
43. Ikeguchi, Y., Wang, X., McCloskey, D. E., Coleman, C. S., Nelson, P., Hu, G., Shantz, L. M., and Pegg, A. E. (2004) *Biochem. J.* **381,** 701–707
44. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. (1997) *FEBS Lett.* **407,** 313–319
45. Sawicki, J. A., Morris, R. J., Monks, B., Sakai, K., and Miyazaki, J.-I. (1998) *Exp. Cell Res.* **244,** 367–369
46. Kato, M., Yamanouchi, K., Ikawa, M., Okabe, M., Naito, K., and Tojo, H. (1999) *Mol. Reprod. Dev.* **54,** 43–48
47. Seiler, N., and Knödgen, B. (1985) *J. Chromatogr.* **339,** 45–57
48. Casimiro, M. C., Knollmann, B. C., Yamoah, E. N., Nie, L., Vary, J. C., Jr., Sirenko, S. G., Greene, A. E., Grinberg, A., Huang, S. P., Ebert, S. N., and Pfeifer, K. (2004) *Genomics* **84,** 555–564

[3] D. E. McCloskey and A. E. Pegg, unpublished observations.

49. Dou, H., Vazquez, A. E., Namkung, Y., Chu, H., Cardell, E. L., Nie, L., Parson, S., Shin, H. S., and Yamoah, E. N. (2004) *J. Assoc. Res. Otolaryngol.* **5,** 215–226
50. Flagella, M., Clarke, L. L., Miller, M. L., Erway, L. C., Giannella, R. A., Andringa, A., Gawenis, L. R., Kramer, J., Duffy, J. J., Doetschman, T., Lorenz, J. N., Yamoah, E. N., Cardell, E. L., and Shull, G. E. (1999) *J. Biol. Chem.* **274,** 26946–26955
51. Tabor, C. W., and Rosenthal, S. M. (1956) *J. Pharmacol. Exp. Ther.* **116,** 139–155
52. Wallace, H. M., Fraser, A. V., and Hughes, A. (2003) *Biochem. J.* **376,** 1–14
53. Hoshino, K., Momiyama, E., Yoshida, K., Nishimura, K., Sakai, S., Toida, T., Kashiwagi, K., and Igarashi, K. (2005) *J. Biol. Chem.* **280,** 42801–42808
54. Pegg, A. E. (2006) *J. Biol. Chem.* **281,** 14529–14532
55. Fong, L. Y. Y., Pegg, A. E., and Magee, P. N. (1998) *Cancer Res.* **58,** 5380–5388
56. Feith, D. J., Bol, D. K., Carboni, J. M., Lynch, M. J., Sass-Kuhn, S., Shoop, P. L., and Shantz, L. M. (2005) *Cancer Res.* **65,** 572–578
57. Eicher, E. M., Southard, J. L., Scriver, C. R., and Glorieux, F. H. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 4667–4671
58. Strom, T., Francis, F., Lorenz, B., Böddrich, A., Econs, M., Lehrach, H., and Meitinger, T. (1997) *Hum. Mol. Genet.* **6,** 165–171
59. Phillips, L. R., and Nichols, C. G. (2003) *J. Gen. Physiol.* **122,** 795–804
60. Kurata, H. T., Phillips, L. R., Rose, T., Loussouarn, G., Herlitze, S., Fritzenschaft, H., Enkvetchakul, D., Nichols, C. G., and Baukrowitz, T. (2004) *J. Gen. Physiol.* **124,** 541–554
61. Williamson, R. E., Darrow, K. N., Michaud, S., Jacobs, J. S., Jones, M. C., Eberl, D. F., Maas, R. L., Liberman, M. C., and Morton, C. C. (2007) *Am. J. Med. Genet. Part A* **143A,** 1630–1639
62. Pegg, A. E., and Williams-Ashman, H. G. (1969) *Biochem. J.* **115,** 241–247
63. Pajula, R.-L., Raina, A., and Eloranta, T. (1979) *Eur. J. Biochem.* **101,** 619–626
64. Wu, H., Min, J., Zeng, H., McCloskey, D. E., Ikeguchi, Y., Loppnau, P., Michael, A. J., Pegg, A. E., and Plotnikov, A. N. (2008) *J. Biol. Chem.* **283,** 16135–16146
65. Basu, I., Cordovano, G., Das, I., Belbin, T. J., Guha, C., and Schramm, V. L. (2007) *J. Biol. Chem.* **282,** 21477–21486
66. Pace, A. J., Lee, E., Athirakul, K., Coffman, T. M., O'Brien, D. A., and Koller, B. H. (2000) *J. Clin. Investig.* **105,** 441–450
67. Delpire, E., and Mount, D. B. (2002) *Annu. Rev. Physiol.* **64,** 803–843
68. Danzin, C., and Mamont, P. S. (1987) in *Inhibition of Polyamine Metabolism. Biological Significance and Basis for New Therapies* (McCann, P. P., Pegg, A. E., and Sjoerdsma, A., eds.) pp. 141–164, Academic Press, Orlando, FL
69. Marton, L. J., and Pegg, A. E. (1995) *Annu. Rev. Pharmacol.* **35,** 55–91
70. Mamont, P. S., Danzin, C., Wagner, J., Siat, M., Joder-Ohlenbusch, A., and Claverie, N. (1982) *Eur. J. Biochem.* **123,** 499–504
71. Pegg, A. E. (1984) *Biochem. J.* **224,** 29–38
72. Haegele, K. D., Splinter, T. A. W., Romjin, J. C., Schechter, P. J., and Sjoerdsma, A. (1987) *Cancer Res.* **47,** 890–895