THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 38, Issue of September 17, pp. 39645–39652, 2004
Printed in U.S.A.

A Spontaneous Point Mutation Produces Monoamine Oxidase A/B Knock-out Mice with Greatly Elevated Monoamines and Anxiety-like Behavior* ♦

Received for publication, May 18, 2004, and in revised form, July 12, 2004
Published, JBC Papers in Press, July 22, 2004, DOI 10.1074/jbc.M405550200

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A spontaneous monoamine oxidase A (MAO A) mutation (A863T) in exon 8 introduced a premature stop codon, which produced MAO A/B double knock-out (KO) mice in a MAO B KO mouse colony. This mutation caused a nonsense-mediated mRNA decay and resulted in the absence of MAO A transcript, protein, and catalytic activity and abrogates a DraI restriction site. The MAO A/B KO mice showed reduced body weight compared with wild type mice. Brain levels of serotonin, norepinephrine, dopamine, and phenylethylamine increased, and serotonin metabolite 5-hydroxyindoleacetic acid levels decreased, to a much greater degree than in either MAO A or B single KO mice. Observed chase/escape and anxiety-like behavior in the MAO A/B KO mice, different from MAO A or B single KO mice, suggest that varying monoamine levels result in both a unique biochemical and behavioral phenotype. These mice will be useful models for studying the molecular basis of disorders associated with abnormal monoamine neurotransmitters.

Two monoamine oxidase (MAO,[1] EC 1.4.3.4.) isoenzymes (MAO A and MAO B) exist closely linked in opposite orientation on the X chromosome (1–3) and are expressed on the outer mitochondrial membrane. MAO A and MAO B oxidize neurotransmitters and dietary amines, the regulation of which is important in maintaining normal mental states. MAO A and B have different substrate specificities. MAO A prefers serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE), and dopamine (DA) as substrates. MAO B prefers phenylethylamine (PEA) as a substrate (for review, see Ref. 4). They are coded by different genes, with 70% amino acid identity (5) and with identical intron-exon organization next to each other on the X chromosome (6). The overall three-dimensional structure of MAO A and B are similar (7), but the mitochondria targeting is different (8). The crystal structure of MAO B is now available (9). The substrate and inhibitor specificities are influenced by a single amino acid (10). The regulations of these two genes are different (11–13). MAO inhibitors have long been used as antidepressant drugs (14), and MAO B inhibitors are used to treat Parkinson's disease (15). Low levels of MAO activity or genetic mutations that abrogate MAO A expression are associated with violent, criminal, or impulsive behavior in humans (16, 17). A recent report on maltreated male children indicates that a variable number tandem repeat polymorphism of the MAO A promoter (four repeats) is associated with less antisocial behavior compared with maltreated children with three repeats in MAO A polymorphism (18). Loss of function of one or both isoenzymes takes place in some forms of Norrie disease marked by mental retardation (19). However, Norrie disease involves multiple gene deletions of the X chromosome, and it is not clear what role MAO deletion may play in this disorder or if Norrie disease provides human model for studying the role of MAO in neurotransmitter metabolism *in vivo*.

Experiments on MAO A or B KO mice (MAO A KO or MAO B KO) mice indicate that absence of each isoenzyme results in a specific biochemical and behavioral phenotype. MAO A KO mice have increased 5-HT, NE, and DA levels and decreased levels of the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) (20), reflecting the preference of MAO A for oxidation of 5-HT. MAO B KO mice have elevated PEA levels, reflecting the preferred substrate of MAO B specificity for PEA (21). MAO A KO mice show increased aggressive behavior (20). MAO B KO mice do not exhibit increased aggressive behavior (21), indicating that the increase in 5-HT, a preferred substrate for MAO A, and concomitant decrease in 5-HIAA may form the basis for increased aggression, consistent with the association of low 5-HIAA levels in the cerebrospinal fluid of men who exhibit aggressive behavior (22, 23). Although increased aggressive behavior has not been observed in MAO B KO mice (21), low platelet MAO B activity in humans is associated with, and considered a marker for, criminal or impulsive behavior (24), although whether this is accompanied in human subjects by a concomitant decrease in MAO A activity or other related genetic or biochemical aberration is not known.

MAO A/B KO mice cannot be generated through the breeding of MAO A KO and MAO B KO mice, due to the close proximity of the isoenzyme genes on the X chromosomes, where the two genes are next to each other at their 3′ tails, organized in opposite orientations with their last exons being less than 24 kb apart (determined by blat analysis of human and mouse MAO A and B at University of California, Santa Cruz Genome

* This work was supported by National Institute of Mental Health Grant R37 MH38635 (Merit Award) and the Elsie Welin Professorship (to J. C. S.) and National Institute of Mental Health Grant RO1 MH NS62148 (to D. P. H.). 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.

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[1] The abbreviations used are: MAO, monoamine oxidase; 5-HT, 5-hydroxytyptamine; NE, norepinephrine; DA, dopamine; PEA, phenylethylamine; 5-HIAA, 5-hydroxyindole acetic acid; KO, knock-out; WT, wild type; HPLC, high performance liquid chromatography.

Server, genome.ucsc.edu). We have identified, bred, and characterized a line of MAO A/B KO mice, which arose by spontaneous point mutation in MAO A exon 8, in a litter of MAO B KO mice. The mutation is very similar to the MAO A point mutation observed in a Dutch family (17), which also occurred in exon 8. The mice exhibit unique biochemical, molecular, and behavioral characteristics.

MATERIALS AND METHODS

Breeding of MAO A/B Double KO Mice—The MAO A/B KO male, progenitor mouse was initially identified by a phenotype characterized by a marked decrease in body weight, behavioral hyper-reactivity on handling, and panic jumping after disturbance (door opening), a phenotype not seen in the MAO B KO mice colony (21). Breeding of the progenitor male with 129/SvEv female mice resulted in a F1 generation that showed no apparent behavioral abnormalities. Female F1 mice were backcrossed with 129/SvEv males. Two types of F2 males were observed: smaller hyper-reactive and larger non-hyper-reactive mice consistent with X-chromosome transmission. Mice with low body weights and hyper-reactive phenotypes were expanded and subsequently were shown to have the MAO A/B double KO phenotype.

Our experiments employed 2–5-month-old, male mice deficient in MAO A/B (MAO A/B KO) and their wild type littermates (WT). The background strain of the animals was that of the MAO B KO mice (21), which had been originally generated in a C57-BL/6J/129Sv strain, whose males were subsequently backcrossed over 25 generations with 129/SvEv females. Animals were singly housed with contact bedding and *ad libitum* food and water. A 24-h diurnal cycle was maintained with lights on from 07:00 to 19:00 h each day. The animal breeding and all experiments performed were approved by the Institutional Animal Use and Care Committee.

Identification of the Site of the Mutation—Liver genomic DNA from MAO A/B KO and wild type mice was isolated using a DNA extraction Kit (Stratagene). PCR amplification of the 15 coding exons of the MAO A gene was performed using the primers designed from the intron sequence flanking the coding region of each exon (Table I). The PCR products were cloned into a pCR4-topo sequencing vector for sequence analysis.

Biochemical Characterization—Absence of MAO A mRNA in these animals was demonstrated by Northern blot using a 1.5-kb mouse MAO A-specific cDNA probe containing the coding region (5). Human MAO A-deficient (male, −) and wild type (female, +/+) fibroblast cells were treated for 2 h with the protein inhibitors puromycin (30 μg/ml) or cycloheximide (100 μg/ml) and then analyzed by Northern blot. Western blot analysis using a MAO A-specific rabbit polyclonal antibody against human MAO A confirmed the absence of MAO A protein using a previously published method (26). MAO assays were performed in duplicate on mouse brain homogenates as described previously (27) using [^{14}C]5-hydroxytryptamine (1 mM) and [^{14}C]phenylethylamine (10 μM) as substrates for estimating MAO A and MAO B activity, respectively.

Determination of NE, DA, and 5-HT levels in brain tissue has been described (28). Whole brains were homogenized in a solution containing 0.1 M trichloroacetic acid, 10 mM sodium acetate, and 0.1 mM EDTA (pH 3.75); 1 μM isoproterenol was used as an internal standard. The homogenates were sonicated and centrifuged, and the supernatants were used for high performance liquid chromatography (HPLC) analysis. 5-HT, NE, DA, 5-HIAA, and 3,4-dihydroxyphenylacetic acid (Sigma) were used as standards. The protein concentrations were determined using the pellet with the method of Lowry (29) with bovine serum albumin as a standard. The mobile phase was the same as the homogenization buffer (excluding the isoproterenol) with 15% methanol for detection of 5-HT. NE was quantified separately using 5% methanol in the trichloroacetic acid mobile phase solution. The mobile phases were filtered and deaerated, and the pump speed (Shimadzu LC-6A liquid chromatograph) was 1.5 ml/min. The reverse-phase column used was a Rexchrom S50100-ODS C18 column with a length of 25 cm and an internal diameter of 4.6 mm (Regis, Morton Grove, IL). The compounds were measured at +0.7 V using a Shimadzu L-ECD-6A electrochemical detector.

PEA was determined as reported previously (21). Briefly, brains excised from mutant and WT mice were homogenized in nine volumes of 0.5 N perchloric acid solution by sonication. Before homogenization, 10 ng of deuterated PEA was added to the samples as an internal standard. PEA was extracted from the homogenate with ether and derivatized with pentafluoroproprionic anhydride. A Hewlett-Packard 5890 gas chromatograph, directly interfaced with a HP5971A mass-selective detector, was used to separate and analyze PEA and the internal standard. Base peaks at 104 and 107 *m/z* were used for detection of PEA and the internal standard, respectively.

Open Field Test—Locomotor activity was measured in a circular arena (43-cm diameter) under indirect lighting over 20 min. Data were collected by video camera and computer interface (Ethovision, Noldus, Inc., Sterling, VA). For each animal, the number of transitions between a peripheral zone (annulus of 8.9-cm width) and a central zone (25.4-cm diameter) were measured, as well as the time (in seconds) spent in the central zone. Group averages were compared using *t* tests (two-tailed, $p < 0.05$). Path length (cm) traveled in the arena was summed for each animal during each minute. A repeated measures analysis of variance was performed using "genotype" as a between subject factor and "time" as a within subjects factor. Path length for each animal was fitted with a random effects exponential model $y = c + m \cdot e^{-k \cdot x}$, where y = path length, m = ordinate intercept, k = rate of decline of locomotor activity, x = time, and c = asymptotic final path length traveled in each minute interval (30). Group differences in the parameters of the equation were tested by *t* test (two-tailed, $p < 0.05$).

Elevated Plus-maze—Standard procedure was used (31) with 5-min test duration, during which time the animal was filmed by a ceiling-mounted camera. Recordings were scored by a blinded observer for time spent on the open and closed arms, using the Tufts Event Scoring System software (Princeton Economics). Entry into an arm of the maze was defined by placement of at least three paws into that compartment. Group averages were calculated for the number of entries and the time spent in open and closed arms of the maze, as well as the total number of rearing events. Genotypic differences were compared using *t* tests (two-tailed, $p < 0.05$).

Social Interaction—Methods were adapted from our previous work (22). After being weaned from their mothers on postnatal day 21, mice were housed singly for 4 weeks in transparent Makrolon cages. A novel intruder mouse was introduced into the cage for 10 min, and the interactions of the mice were videotaped. Intruders were weight-matched male mice of the same genotype as the resident animal. Social behavior was coded from video recording using Tufts Event Scoring software by a blinded observer according to standard definitions (32, 33), which are: 1) non-social (absence of exploration of other mouse), 2) investigative (subject actively investigating the cage, mostly by sniffing), 3) aggressive (biting, lateral attack, tail rattling, or climbing on top of the intruder), 4) chase/escape. Chasing was scored separately to emphasize the fact that aggressive encounters in the MAO A/B KO mice were characterized predominantly by chasing and less so by other aggressive behaviors. Latency to attack was recorded and included any aggressive encounter of the resident with the intruder, including chasing, biting, lunging, on the top behavior, but not simple tail rattle. Genotypic differences were analyzed by *t* test (two-tailed, $p < 0.05$).

Home Cage Locomotor Activity—MAO A/B KO mice (n = 8, age = 16.8 ± 0.4 weeks) and WT mice (n = 11, age = 16.5 ± 0.3 weeks) received an intraperitoneal radiotransmitter implant (model TA10ETA-F20, Datasciences International) using methods reported previously (34). Beginning 2 weeks postsurgery, locomotor activity was recorded in the home cage of the animal in 10 s segments every 3 min over 7 days. Activity counts were separately summed for each animal during the light phase (07:00 to 19:00) and the dark phase (19:00 to 7:00) across the 7-day period. Statistical comparison of genotypic differences was performed with a repeated measures analysis of variance, and *post hoc t* tests (two-tailed, $p < 0.05$) were used to examine genotypic differences during each 12-h light/dark cycle.

RESULTS

Greatly Elevated Monoamine Levels in MAO A/B KO Mice—The MAO A/B KO mouse was initially identified by observing a mouse in a litter of MAO B KO mice, previously generated by homologous recombination (21), of markedly lower body weight (Fig. 1*A*), which exhibited extreme behavioral hyper-reactivity triggered by the approach of the experimenter to the cage of the animal, which resulted in an exaggerated escape response. The body weight and behavior were inconsistent with the phenotype of previously characterized MAO B KO mice (21).

Breeding of the hyper-reactive, low body weight mouse revealed an X-linked transmission, consistent with the X linkage of MAO A and B genes (2). HPLC analysis of urine demonstrated non-detectable levels of the MAO A metabolite 5-HIAA in the hyper-reactive, low body weight mice. In the MAO A/B KO hyper-reactive, low body weight mice, 5-HIAA levels were decreased to even lower levels than seen in MAO A KO mice (Fig. 1*B*). Enzymatic activity in brain was then assessed and

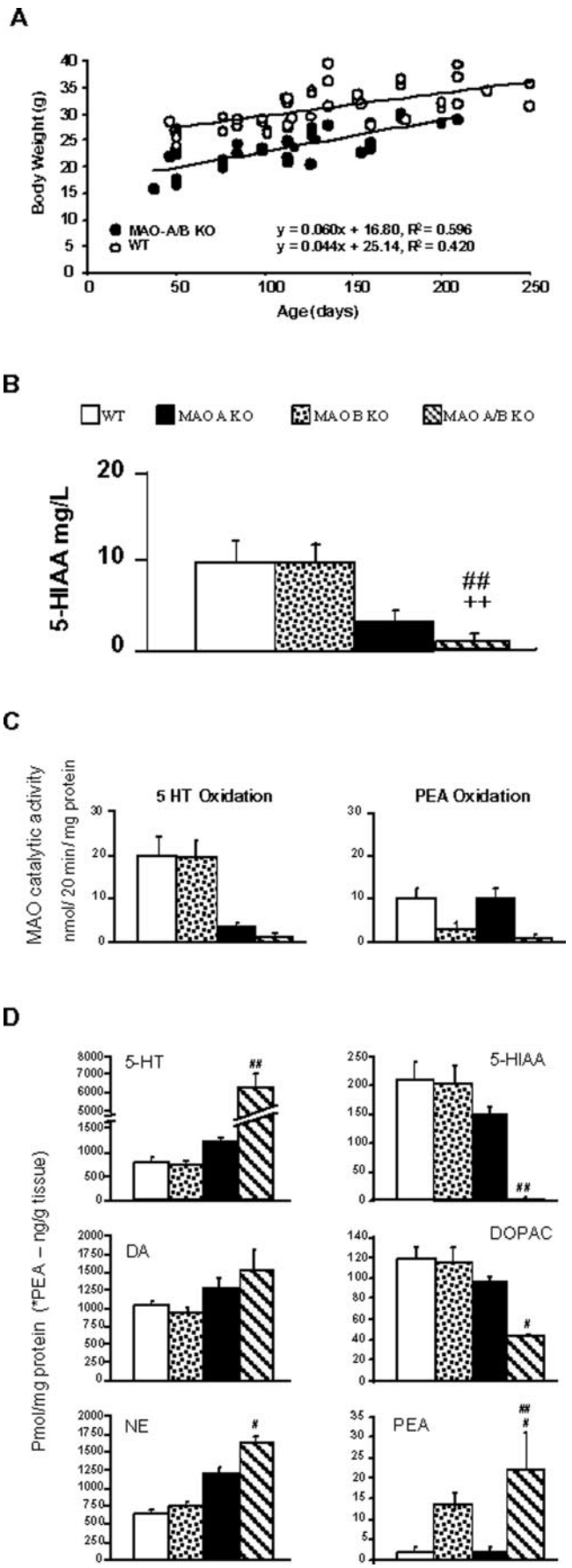

FIG. 1. *A*, comparison of body weights of MAO A/B KO mice and wild type mice with respect to age in days. Significant differences were examined using an analysis of covariance, in which age was used as a covariate factor. Mutant animals ($n = 32$) showed a significant decrease in body weight ($F_{1,69} = 540.38$, $p < 0.00001$) compared with controls ($n = 40$), with the mean body weight (±S.E.) for MAO A/B KO mice being 24.15 ± 0.49 g and that for WT mice being 30.58 ± 0.44 g. *B*, 5-HIAA urine levels. Values are expressed in mg/liter and represent the mean ± S.E. *n* represents the number of mice. Unpaired *t* test for MAO B wild type *versus* MAO A/B double KO mice (##, $p < 0.01$) and MAO A KO *versus* MAO A/B double KO mice (++, $p < 0.01$): wild type ($n = 2$), MAO A KO ($n = 3$), MAO B KO ($n = 4$), MAO A/B double KO ($n = 7$). *C*, MAO A and B catalytic activity determined by the rate of oxidation of their substrates, 5-HT and PEA, respectively (nmol/20 min/mg of protein). In MAO A/B KO counts/min levels for both substrates were similar to background. *D*, MAO substrate and metabolite levels in mouse brain homogenate. Mice are designated as in *B*. All values except PEA are expressed in pmol/mg of protein and represent the mean ± S.E. MAO A/B wild type and MAO A/B double KO mice ($n = 4$, each). Unpaired *t* test comparing MAO A KO *versus* MAO A/B double KO mice show significant changes in 5-HT, 5-HIAA (##, $p < 0.01$), and 3,4-dihydroxyphenylacetic acid (#, $p < 0.05$). PEA values are expressed in ng of PEA/g of tissue and represent the mean ± S.E. Unpaired *t* test for MAO B wild type *versus* MAO A/B double KO mice (##, $p < 0.01$) and MAO B KO *versus* MAO A/B double KO mice (#, $p < 0.05$). Data for MAO B KO mice were taken from Grimsby *et al.* (1997).

the results indicated a complete loss of both MAO A and MAO B activity (Fig. 1*C*), as measured by oxidation of 5-HT and PEA, respectively.

Next, we examined the levels of monoamine neurotransmitters oxidized by the MAO isoenzymes, as well as PEA levels and neurotransmitter metabolites in brain homogenates of MAO A/B KO mice. PEA and neurotransmitter levels were increased over wild type. However, more importantly, MAO substrate levels were increased, and metabolite levels were decreased, compared with those measured in each of the single MAO A or B KO mice (Fig. 1*D*), as assessed by HPLC or by gas chromatography/mass spectrometry in the case of PEA. In MAO A/B KO mice 5-HT, NE, DA, and PEA levels were elevated 8.5-, 2.2-, 1.7-, and 15.7-fold, respectively, above those in WT animals. Although elevated 5-HT and PEA levels are, respectively, consistent with an absence of the MAO A or MAO B isoenzyme, the magnitudes of either 5-HT or PEA increases are much greater than in single MAO isoenzyme KO mice. Since NE and DA can also be metabolized by catechol-*o*-methyl transferase, a less extreme increase of their levels in brain compared with 5-HT or PEA was seen in the hyper-reactive, low body weight mice or in previously reported single KO MAO mice (20, 21). 5-HIAA levels in brain homogenates were decreased compared with the already greatly reduced levels in the MAO A KO mice, and decreases of 5-HIAA levels were about 200-fold less than those of WT mice or MAO B KO mice (Fig. 1*D*). MAO B expression increases with age, and consequently, in MAO A KO mice, increases of 5-HT and decreases of 5-HIAA become less pronounced in adult and aged mice (20). MAO B is generally considered to be absent in newborn mice, as assessed by current MAO assays, ostensibly making a newborn MAO A KO mouse very similar if not equivalent to the double MAO A/B KO mouse in terms of MAO expression. Yet, MAO A/B KO mice have increased 5-HT and decreased 5-HIAA levels compared with newborn MAO A KO mice (20). These data suggest that even newborn mice may have a basal level of MAO B activity and that the MAO A/B double KO has all MAO activity abrogated.

$A^{863} \rightarrow T$ Mutation in MAO A Gene Results in Nonsense-mediated mRNA Decay and MAO A/B Double KO Mice from MAO B KO Mice—Given the implications of the above-described altered biochemical phenotype of the low body weight, hyper-reactive mice observed and bred from the MAO B KO litter, the presence or absence of MAO A transcript and protein were assessed by Northern and Western blot, respectively. No observation of transcript by Northern analysis (Fig. 2*A*), nor protein by Western blot, demonstrated the absence of MAO A

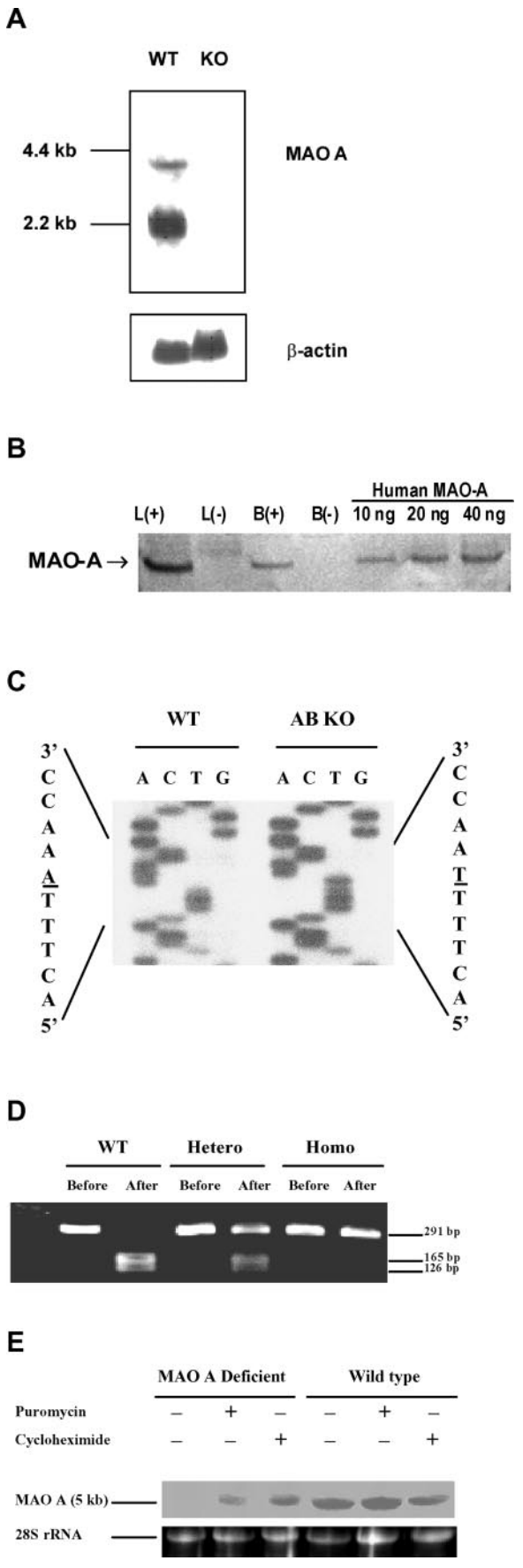

FIG. 2. *A*, Northern blot of MAO A mRNA expression in wild type (*WT*, n = 2) and MAO A/B KO mice (*KO*, *n* = 2). Thirty and fifty micrograms of total RNA from WT and MAO A/B KO brain extracts were loaded, respectively, for each sample and were probed with a 1.5-kb mouse MAO A-specific cDNA probe containing the coding region (*top*). The same blot was probed with β-actin (*bottom*). *B*, Western blot analysis of MAO A in outer membrane mitochondrial extracts from liver (*L*) and brain (*B*) of MAO B KO (+) and MAO A/B KO (−) mice. Comparison is made to purified MAO A protein (10–40-ng samples). The *arrow* on the *left side* indicates the position of the MAO A protein band. This is a representative of four experiments for each genotype. *C*, nonsense mutation A863T was found in exon 8 of MAO A/B KO mice (base substitution marked by *underlines*). The DNA sequence of all the other 14 exons are identical between MAO A/B KO mice and WT. *D*, PCR products before and after DraI digestions of exon 8 genomic DNAs from MAO WT, heterozygous MAO A/B KO,and homozygous MAO A/B KO mice. *E*, detection of aberrant mRNA of mutated MAO A gene after the inhibition of protein synthesis. Human MAO A-eficient (−) and wild type (+) fibroblast cells were treated with protein inhibitors puromycin (30 μg/ml) or cycloheximide (100 μg/ml) for 2 h and then analyzed by Northern blot.

TABLE I
Primers used for sequencing each exon in mouse MAO A gene

Name	Position	Primers (5′→3′)
Exon 1		
P1255	Promoter	CGA ATC CCT GTT GCC TAT GT
In1R	Intron 1	TTA TTT TGC CTC CCT CCA CC
Exon 2		
In1F	Intron 1	GTA GAT CTG GGA TGT TTG GC
In2R	Intron 2	ATG AGA GCT CCT CTA GGG C
Exon 3		
In3F	Intron 2	ATT GTC AGA CAG TGT TCT CC
In4R	Intron 3	CCT TAT TTG CAG TTC CTA G
Exon 4		
In4F	Intron 3	CTG CTC GTG CTC CTG CTG TT
In5R	Intron 4	ACA GAC ACA CAG ACA CAC TC
Exon 5		
In4F	Intron 4	CTC CAG ATT CAT CTC ACC TC
In5R	Intron 5	GCA CTA CTA CTC AAG ACA G
Exon 6		
In5F	Intron 5	TAG TGT AGT TAG GTA GTA TC
In6R	Intron 6	GGA TCT AAG GAA TTG GGA
Exon 7		
In6F	Intron 6	CCC AGA GCT CCT TGT AT
In7R	Intron 7	GAA CTC CTG TAT GCT TCC TG
Exon 8		
In7F	Intron 7	ACG CGC TCT TCT GGT GCA T
In8R	Intron 8	AGC TTA CTT CAG GGC
Exon 9		
In8F	Intron 8	GCT TAT CAG GGT GTT GTG
In9R	Intron 9	CTG TCT TTC TAG CTG CTT G
Exon 10		
In9F	Intron 9	TAG CTC ACA GGC TAC AGA GT
In10R	Intron 10	ACA TGT TAG CGG CTA TGA T
Exon 11		
In10F	Intron 10	TTC AAG AAG AGA TGA GCC
In11R	Intron 11	ACG GGA TCT CTG TTC TGC
Exon 12		
In11F	Intron 11	GAA TCT GTA CGA ATG AGA G
In12R	Intron 12	GTC CTG TGA GAC TAA ATG T
Exon 13		
In12F	Intron 12	CCC AAA TCT GAG GAT GT
In13R	Intron 13	GTG AAG GAG ATG ATA ATG
Exon 14		
In13F	Intron 13	GTT GTC ACA TTG ACA GG
In14R	Intron 14	GGT CTG TAG ATA TGG AG
Exon 15		
In14F	Intron 14	GCA CTG TCC TTC ATT TAG CC
In15R	3′UTR	GCA CTT AAA TTG CCC AAA CC

protein (Fig. 2*B*), confirming that the mice were deficient in both MAO A and MAO B expression. The observed loss of MAO A activity was due to a spontaneous mutation in MAO A, creating a double KO for both MAO A and B as was shown in the following experiments.

To determine the molecular basis for the mutation, PCR primers were designed that flanked each of the 15 exons of MAO A (Table I). Exon sequences were amplified from genomic DNA, subcloned, and sequenced. Sequence analysis identified a point mutation in exon 8 where adenine at position 863 of MAO

FIG. 3. **Sites of spontaneous mutation in mouse and human MAO A, exon 8.** *Shadings* indicate sites of mutation resulting in premature stop codons, A863T in mouse (*top*) corresponding to amino acid 284 (lysine; *K*) and C950 in human (*bottom*) corresponding to amino acid 296 (glutamine; *Q*). Nucleic acid numbering is according to mouse MAO A GenBank™ entry NM_173740 and human gene bank entry M68840 as reported in Ref. 5.

A (numbering relative to the mouse MAO A GenBank™ entry NM_173740) is mutated to thymine (Figs. 2*C* and 3). This substitution results in the introduction of a stop codon at amino acid 284 rather than a lysine in wild type (AAA to TAA).

The A863T mutation abrogates a DraI restriction site (TTTAAA → TTTTAA). DraI cleavage patterns of amplified exon 8 were analyzed in wild type, heterozygous, and homozygous mutant mice and resulted in cleavage patterns consistent with the A863T mutation (Fig. 2*D*). Wild type showed normal DraI cleavage, homozygous mutant mice were unaffected by DraI, and heterozygous mice showed both cleavage product and non-cleaved DNA, confirming the sequence results for the PCR amplified exon 8.

The molecular basis for the MAO A deficiency determined in the mice identified as being MAO A/B KO genotype differs from the initially reported MAO A KO mouse, Tg8 (19). The Tg8 MAO A KO mice was based upon an insertion of the interferon-B gene into the MAO A gene with a concomitant deletion of exons two and three of the MAO A gene. Four mRNA species were observed in Tg8 mice (20), none of which resulted in viable MAO A protein, whereas in the A863T point mutation MAO A/B KO mice, which harbors an early termination codon, no mRNA was observed by Northern blot (Fig. 2*A*).

The A863T point mutant is near the MAO A point mutation seen in a Dutch family (17), where cytidine at position 936 (950 using GenBank™ accession number M68840) in exon 8 is mutated to thymine (C936T, numbering as cited in Ref.17, corresponding to GenBank™ entry M69226) to result in an early termination codon at amino acid 296, where normally glutamine is coded for (CAG to TAG). The mutation reported here in the A/B double KO mice was in the same exon 8, 36 nucleotides, or, when translated, 12 amino acids, proximal to the premature stop codon of the human mutant (Fig. 3). The mutation in the Dutch family results in a complete absence of mRNA detectable by Northern analysis which correlates with the absence of mRNA in the MAO A/B KO mice but differs from the four smaller mRNA species observed in Tg8 MAO A KO mice (20). This absence of transcript is dependent on protein synthesis, as pretreatment of human fibroblasts harboring this point mutation with the protein synthesis inhibitors cyclohexamide and puromycin results in the presence of aberrant MAO A transcripts in the cells (Fig. 2*E*). These observations are consistent with nonsense-mediated mRNA decay (35), which protects cells against translation of aberrant transcripts and associated truncated protein products taking place in both the human and mouse point mutations but not in the Tg8 mutation. The observed MAO A deficiency described here in the MAO A/B KO mice is thus a spontaneous nonsense mutation and is analogous to, indeed almost identical to, the human mutation observed in males of a Dutch family who exhibit impulsively aggressive or anti-social behavior.

MAO A/B Knock-out Mice Show Anxiety-like Behavior—Behavior of the MAO A/B KO mice was examined and a unique phenotype exhibiting anxious traits was observed. MAO A/B KO mice displayed less exploratory activity in an unfamiliar open field than WT mice ($F_{1,17} = 22.13$, $p < 0.0005$) (Fig. 4*A*). The asymptotic final distance traveled in each minute interval was significantly lower in MAO A/B KO mice (43.51 ± 11.53 cm min^{-1}) than in WT mice (93.45 ± 13.67 cm min^{-1}, $p < 0.01$), and the initial slope of decline of locomotor activity was significantly greater in MAO A/B KO mice (1.60 ± 0.35 cm min^{-2}) than in WT mice (0.51 ± 0.05 cm min^{-2}, $p < 0.02$). Time spent in the central, most exposed portion of the arena was significantly less ($p < 0.05$) in MAO A/B KO mice than in WT mice (Fig. 4*B*). This decreased exploratory behavior was not due to decreased basal levels of locomotor activity, as there was no genotypic difference in diurnal activity variation (dark phase: MAO A/B KO, 3,348 ± 143 counts/12 h; WT, 3,552 ± 293 counts/12 h and light phase: MAO A/B KO, 2,199 ± 136 counts/12 h; WT, 2144 ± 167 counts/12 h) whether activity was compared across the dark phases, the light phases, or across both phases ($p > 0.05$) for each day or across a 7-day period. This pattern in MAO A/B KO mice of locomotor inhibition and avoidance of the center of the arena reflected a behavior consistent with increased anxiety. In contrast, MAO A KO mice do not exhibit similar avoidance of the center, most exposed area in the open field test (20).

In the elevated Plus-maze (31), another behavioral paradigm to assess anxiety, MAO A/B KO mice behavior was characterized by freezing or crouching postures, during which time the animals remained immobile. Consistent with this, MAO A/B KO compared with WT mice demonstrated a smaller number of entries into both the open and closed arms of the maze (Fig. 4*C*, $p < 0.001$), as well as fewer rearing events (Fig. 4*D*, $p < 0.01$). The percentage of total entries made by MAO A/B KO mice into closed arms was significantly higher, and that into open arms was significantly lower, compared with entries made by WT mice (Fig. 4*E*, $p < 0.002$). Likewise, MAO A/B KO mice spent more time in the enclosed arms than in the open arms of the maze (Fig. 4*F*, $p < 0.001$). This pattern differed significantly ($p < 0.001$) from that of WT mice, which showed little preference between the two arms (Fig. 4*F*, $p > 0.05$). The pattern of behavior in which ongoing activity is inhibited and exploration is preferentially directed toward closed arms of the maze is consistent with anxious, avoidant behavior, as opposed to active exploration of the environment. Again, this is not congruent with observations in either the MAO A KO mice (20, 36) or the MAO B KO mice (21), whose behavior in the elevated

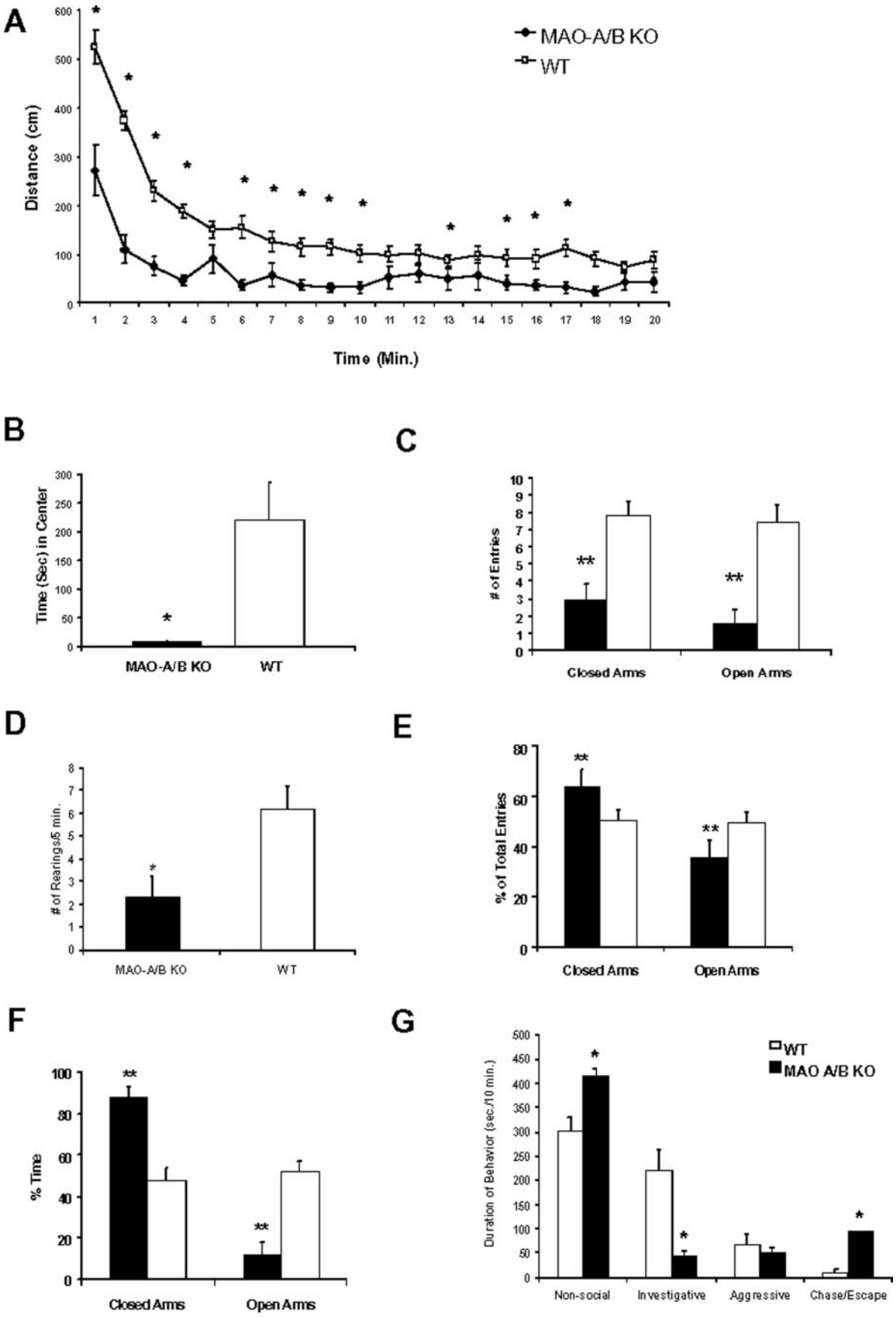

FIG. 4. Exploratory locomotor activity in a novel arena (*A*, *B*). Depicted are mean values (±S.E.) of MAO A/B KO mice (♦, $n = 8$) compared with WT mice (□, $n = 11$). MAO A/B KO mice show a significantly decreased (*A*) distance traveled in the arena, as well as (*B*) time (in seconds) spent during the 20-min trial in the central part of the arenal; *, $p < 0.05$. Elevated Plus-maze (*C–F*), results are shown for MAO A/B KO mice (*black columns*, $n = 13$, age = 17.8 ± 2.5 weeks) compared with wild type mice (*white columns*, $n = 18$, age = 19.2 ± 2.1 weeks). Depicted are number of entries (*C*) (placement of at least three paws into an arm of the maze), number of times animal adopted a rearing posture (*D*), percentage

Plus-maze does not significantly differ from that observed in WT mice.

Social interaction was assessed with the resident-intruder paradigm, in which an unfamiliar intruder mouse is introduced into the home cage of a mouse previously singly housed for 4 weeks. MAO A/B KO mice compared with WT mice demonstrated significant increases in non-social behavior ($p < 0.01$) and significant decreases in investigative behavior ($p < 0.01$) (Fig. 4*G*). Behaviors in the mutant mice were interrupted by a significant increase in rapid chase and escape responses ($p < 0.01$) (Fig. 4*G*). Latency to attack was significantly less in MAO A/B KO mice (15.5 ± 6.0 s.) than in WT mice (533.3 ± 39.5 s, $p < 0.001$), with the decreased latency of mutant animals primarily related to a chasing of the intruder, which occurred almost immediately on first encounter. Chases were extremely rapid and would terminate either by brief physical aggressive contact or on occasion by animals jumping against the cage walls. Typical aggressive behaviors such as biting, lateral attack, tail rattle, on-the-back behaviors, and offensive upright postures, however, did not change significantly in the MAO A/B KO mice (Fig. 4*G*). Thus, classical attack sequences did not as clearly establish themselves in the mutant compared with the wild type mice because of the hyper-reactivity of the animals and the extensive pursuit, as well as escape.

DISCUSSION

The MAO A point mutation described for the MAO A/B KO mice differs from prior MAO A KO mice that were generated serendipitously by a random integration of a gene, which had been introduced to create transgenic mice, within the MAO A gene (20). The question arises as to what drove this spontaneous point mutation in the MAO B KO mice? Of note is that this mutation occurred in two mice out of a total 600 MAO B KO mice, which is not an infrequent occurrence, indicating that there is a driving force behind the mutation beyond pure randomness. MAO B KO mice have greatly elevated levels of PEA, indicating that PEA or a metabolite is a possible candidate for mediating the mutagenic response. Of interest in this respect is a report that rabbit microsomes can convert PEA into azoxy-2-phenylethane, which demonstrates mutagenic activity consistent with a point mutation in Ames salmonella test systems (37). Similarly, PEA can be *p*-hydroxylated to become tyramine (38), which when nitrosated becomes genotoxic (39). It is conceivable that the high concentrations of PEA resulting from MAO B KO mutation shifts an equilibrium, which allows production of unusually high levels of azoxy-2-phenylethane or nitrosated tyramine. Future work should examine an association between high levels of PEA in individuals with low platelet MAO B activity and potential MAO A mutations or mutations in related catecholaminergic systems such as the 5-HT transporter (40).

Anxiety-like behavior was prominent in the MAO A/B KO mice and significantly greater than in the 129/Sv wild type mice, which as a strain may themselves show increases in basal levels of anxiety compared with other strains (41, 42). Since the monoamines 5-HT, NE, DA, and PEA are all elevated in the MAO A/B KO mice, it is difficult to pinpoint which monoamine is primarily responsible for the observed behavior, particularly since all these amines have anxiogenic properties. A central function in the control of anxiety has been ascribed to 5-HT, with 5-HT_{1A}, 5-HT_2, and 5-HT_3 receptor subtypes playing roles of differing importance (43–45). Research has also delineated a central function to the noradrenergic system in the mediation of anxiety, particularly to the acquisition of conditioned fear (46). The dopaminergic system may also be involved in anxiety disorders, in particular those where dopaminergic activation involves both D_1 and D_2 receptors (47). Likewise, multiple neurotransmitters have also been implicated in the regulation of body weight (48) and social behavior (49, 50).

Both increases and decreases in 5-HT content of the brain have been associated with anxiety in KO mice. Increased anxiety is seen in mice lacking the 5-HT transporter (42) and in mice lacking the 5-HT transcription factor PET-1 (51), both in association with either low or nearly absent cerebral 5-HT. In contrast, MAO AB KO mice show anxiety in association with increased cerebral serotonin levels. A similar situation exists for aggression. PET-1 knock-outs and MAO A knock-out show increased aggression, but they have opposite serotonergic profiles. This suggests the possibility of a U-shaped relationship between anxiety (or aggression) and serotonergic levels.

What is becoming increasingly clear for alterations in any single neurotransmitter is that the phenotype of an animal is determined by actions at multiple receptor subtypes (52, 53), with different behavioral consequences in different brain regions (54). Furthermore, developmental adaptations during brain maturation in knock-out mice may result in a phenotype that is paradoxically different from that elicited by acute pharmacologic intervention in an adult wild type animal. Thus, for example, phenylethylamine when administered pharmacologically to rodents has been found to exert strong amphetamine-like effects, resulting in increased anxiety and locomotor activity (55). An argument against the importance of PEA in anxiety-like behavior of MAO A/B KO mice is the fact that mice with a single MAO B knock-out mutation display markedly elevated PEA levels, without elevations in 5-HT, NE, or DA but show no evidence of heightened anxiety either subjectively or as tested in the elevated Plus-maze (21). It is possible that such a paradoxical finding may be the result of some form of developmental compensation in the MAO B KO mice.

Given the complex relationship between multiple neurotransmitter systems and anxiety-like behavior, the question arises as to the differences in behavioral phenotype between MAO A/B KO and MAO A KO mice. Subjectively, the MAO A KO mutants are less anxious and more aggressive. MAO A/B KO mice, like MAO A KO mice (20), display increased aggressive behavior in the resident-intruder paradigm; however, aggression is characterized largely by chasing of the intruder rather than distinct biting, tail rattling, upright offensive postures, or lateral attack sequences. The differences in levels of anxiety between MAO A/B KO and MAO A KO mice, as well as the differences in aggressive display may reflect different underlying biochemical profiles of these animals. This suggests that elicitation of specific behaviors such as anxiety may depend on the levels of neurotransmitters and that the presence of anxiety may shape the expression of aggression. As has been proposed by others (56, 57), aggression and anxiety may be conceptualized as part of a continuum of behaviors sensitive to the levels of behavioral arousal elicited, for instance, by increases in brain 5-HT. Differences in the behaviors elicited by such increases in 5-HT may depend on their relative actions at different serotonergic receptor subtypes, which in the case of

of total entries made into closed and open arms (*E*), percentage of time spent in closed and open arms during the 300-s test (*F*). MAO A/B KO *versus* WT mice: *, $p < 0.01$; **, $p < 0.001$. *G*, social interaction during confrontation with a novel intruder. Depicted are means (±S.E.) for MAO A/B KO mice (*black columns*, $n = 8$) and WT mice (*white columns*, $n = 8$). Behaviors over a 10-min observation period were classified by a blinded observer as non-social (absence of exploration of other mouse), 2) investigative (subject actively investigating the cage, mostly by sniffing), 3) aggressive (biting, lateral attack, tail rattling, or climbing on top of the intruder), 4) chase/escape. *, $p < 0.01$.

5-HT_{1A} knock-out mice and 5-HT_{1B} knock-out mice have been associated, respectively, with increased anxiety and increased aggression (25, 58).

In conclusion, a MAO A/B double KO mouse has arisen by a spontaneous point mutation of MAO B KO mice. We have bred the A/B double KO mice and characterized their biochemical, molecular, and behavioral phenotypes, each of which differ from previously created MAO A or MAO B KO mice. The hallmarks of this MAO A/B KO phenotype are decreased levels of 5-HIAA compared with MAO A KO mice, increased levels of PEA, 5-HT, NE, and DA compared with either MAO B KO or MAO A KO mice, and a behavioral phenotype indicating heightened anxiety with less classically aggressive behavior and increased chase/escape responses than single MAO A KO mice.

The availability of three different MAO KO mice (MAO A, MAO B, and MAO A/B) provides a unique opportunity to further examine the molecular details of the monoamine neurochemical systems associated with specific behavior or psychological states. It will also provide new insights for developing selective pharmacological interventions for diseases involving abnormal catecholamine catabolism.

Acknowledgments—We thank Dr. Ken Roos for his technical support and Dr. O. U. Scremin for analytical help. We thank Dr. Han Brunner for human fibroblast cell lines.

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