Supplemental Methods

Gene expression and splicing

TaqMan assay Hs01053458_m1 was used to quantify total *FMO3* mRNA expression (Applied Biosystems, CA, USA). Gene expression levels were assessed by real-time PCR using an ABI-7900HT Fast real-time PCR system. Each reaction was run in duplicate while simultaneously amplifying *GAPDH* (Hs02758991_g1). Total mRNA expression levels were normalized with *GAPDH* mRNA expression to obtain relative mRNA expression levels.

PCR products of the correct size were confirmed for all splicing assay primer pairs (primer sequences, Table S1) by agarose gel electrophoresis. Reactions for pairs of assays to be compared in each experiment were arrayed together in the same 384-well plate in duplicate pairs, and run on an ABI-7900HT Fast real-time PCR system under standard conditions including 2x PerfeCTa SYBR Green FastMix ROX (Quant Biosciences, Gaithersburg, MD). Dissociation curves demonstrated single peaks consistent with little contamination from primer-dimers. Ct values were the average of two reactions for each sample and assay. The difference in relative quantity was determined by subtracting the smaller average Ct value of one reaction from the larger average Ct value of the other reaction.

Microsome Preparation

Microsomes were prepared from pelleted cultured cells or flash-frozen tissues. Tissue in ice-cold potassium phosphate buffer pH 7.4 were homogenized 6x with a glass-teflon homogenizer, centrifuged 20 min at 9,000 *g*. The S9 fraction was centrifuged 60 min at

100,000 *g* to obtain a microsomal pellet which was resuspended, further homogenized 6x, centrifuged another 60 min at 100,000 *g* and finally resuspended and homogenized 6x in potassium phosphate buffer containing 20% glycerol for storage at -80°C.

Microsomal incubations

Incubations were conducted in 96-well plates 50μl volumes containing 100 mM potassium phosphate buffer, pH 8.4, microsomal protein (30-1,500 μg/ml), nicotine (100 μM-5 mM; Toronto Research Chemicals, Toronto Canada) and 1 mM NADPH (Sigma-Aldrich). Following preincubation at 37° C for 3 min, metabolic reactions were initiated by the addition of nicotine and quenched after 6 hours with 15% zinc sulfate containing internal standard (d_3-*trans*-nicotine-*N*-oxide, 10ng/ml for human brain and astrocytes, 2μg/ml for all others). d_3-*trans*-Nicotine-*N*-oxide (TRC) was utilized for quantification of both *cis* and *trans N*-oxide.

Liquid chromatography tandem mass spectrometry

LC-MS\MS analyses were performed on an API 4000 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems Sciex, Foster City, CA) equipped with an electrospray source. The HPLC system consisted of two LC 20AC pumps with a CTO-20A column over, SIL-20A autosampler, DGU-20A3 degasser, FCF-11AL valve, and a CBM 20A controller (Shimadzu, Columbia, MD, USA). The chromatographic separation was performed on an xBridge column (150 x 2.1, 3.5μm, Waters, Milford, MA, USA) with a pre-column inline 0.2 μm filter. The injection volume was 5 μL and the oven temperature was 40 °C. Mobile phase (0.3 ml/min) was (A) 0.1% ammonium formate, pH 4.0 and

(B) methanol using the following program: 1% B for 4 minutes, linear gradient to 25% B between 4 and 5 minutes, held at 25% B until 6.0 minutes, and then re-equilibrated to initial conditions between 6.5 and 8.5 minutes. Under these conditions, the retention times for *cis* and *trans* nicotine-*N*-oxide were 4.69 and 5.23, respectively. The instrument was operated in positive-ion mode at 450 °C with an ion spray voltage of 5500 V, entrance potential of 10 V and exit potential of 22 V. The curtain gas was set at 20, ion source gas 1 at 30, and ion source gas 2 at 40. Transitions monitored for nicotine-*N*-oxide were *m/z* 179→132 and for deuterated (d_3) *m/z* 182→132.

Electrophysiology

Voltage and current electrodes were pulled from capillary glass (OD = 1.20 mm, ID = 0.69 mm), and filled with 3 M KCl. The electrodes typically had resistances of less than 1 MΩ. The oocytes were clamped at -60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT, USA) was perfused continuously at approximately 5 ml min^{-1}. Bath solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES; pH 7.4) was perfused between all test applications. Solutions were gravity-applied from 30 ml glass syringes with glass luer slips via Teflon tubing to reduce adsorption and switched manually.

A typical experiment consisted of recording a 10 sec baseline response followed by a 10-60 sec drug application and a bath application (up to 4 min) until full recovery. The duration of drug application (ACh, *trans*-nicotine-*N*-oxide, or ACh + *trans*-nicotine-*N*-oxide) was dependent on the concentration of drug, and aimed at reaching a saturated peak response without unnecessary further exposure to the drug. The current responses

were amplified with an OC-725C amplifier (Warner Instruments), filtered at 40 Hz, digitized with a Digidata 1200 series digitizer (Molecular Devices, Sunnyvale, CA, USA) at a 100 Hz sampling rate, and stored using pClamp (Molecular Devices). The traces were subsequently analyzed with Clampfit (Molecular Devices) to determine the peak amplitude of current response. Acetylcholine chloride, *trans*-nicotine-*N*-oxide, and inorganic salts from Sigma-Aldrich. Working concentrations of drugs were made on the day of the experiment.