Supplemental Methods and Materials

Non-human primate subjects

The subjects were 14 male rhesus monkeys (Macaca mulatta). They were housed in standard stainless steel cages in a room with a 14:10 light:dark cycle, lights on at 8AM. They weighed between 6.8 and 11.6 kg at the beginning of the study. They ranged in age between 10 and 16.5 years. They were fed daily between 105 and 290 grams of Teklad 25% Monkey Diet (Harlan/Teklad, Madison, WI), individually determined to maintain stable body weight. Water was available ad libitum.

Drug administration and euthanasia procedures

On their final quarantine medical exam, they were anesthetized with ketamine and 3-5 ml of whole blood was collected from each animal for genotyping. Blood samples were stored at -70˚ C until required for PCR. Genomic DNA was extracted from whole blood using a Qiagen QIAmp DNA Blood Maxi Kit following manufacturer's instructions. Isolated genomic DNA was stored at -70˚ C until processed for PCR. The PCR genotyping for the serotonin transporter was done essentially as described by Lesch et al, (1997). One monkey was homozygous SS, four were LS and the rest were LL. Monkeys were divided into two groups, fluoxetine-treated and vehicle-treated (control), counterbalanced to the extent possible for genotype and age (Table S1). The SS monkey was assigned to the treated group, two LS monkeys were assigned to each treated and control groups, and five LL monkeys were assigned to the treated group and four LL monkeys to the control group.

The monkeys were fitted with metal collars and trained over two-three weeks to be removed from the cages and seated in a primate restraint chair (Primate Products, Inc., CITY). This was done to allow blood drawing from unanesthetized monkeys. Next, they were allowed to drink 50 ml of 4% Tang from a 500 ml bottle hung on the front of the cage at approximately 1:00 PM each day. Initially,

Tang bottles were left on the cages until all 50 ml were consumed. Gradually (over several days), Tang availability was shortened to 30 minutes/day. When all Tang was consumed in 30 minutes for at least three consecutive days, pre-drug baseline blood samples were drawn.

Pre-drug blood was collected from each subject prior to daily dosing for determination of trough fluoxetine/norfluoxetine concentrations. Monkeys were seated in chairs (Primate Products) and 4-5 ml of blood was withdrawn from the saphenous vein. After blood drawing, the monkey was returned to his cage. Samples were collected using K_2EDTA Vacutainer® brand tubes. Each sample was centrifuged at 3000 x G for 10 minutes before the plasma was transferred to a 12 x 75mm polypropylene tube. Aliquots of plasma were stored at -70^{o}C for up to one week before analysis as per the stability of fluoxetine (Li et al., 2004).

For analysis, 100 mcL of the internal standard solution, 1 mcg/mL citalopram in H_2O, was added to 1.0 mL of plasma and three mL 0.1M monobasic potassium phosphate buffer, pH 6.0 in a glass reaction tube. Fluoxetine, norfluoxetine, and the internal standard were extracted from plasma via strong cation exchange solid phase extraction as per the method of Wille et al. (2005). Sample extracts were evaporated to dryness using compressed nitrogen before being reconstituted with methanol and injection onto a Waters Alliance HPLC system (Milford, MA). Analytes were separated using a 150 x 4.6mm, 5μ, Aqua C18 column (Phenomenex, Torrance, CA) and detected using a Waters 996 photodiode array detector set to 225 nm. Citalopram, norfluoxetine and, fluoxetine eluted at 3.56, 4.96, and 5.63 minutes respectively (Figure S1). Quantification was performed by comparing the peak area response ratio of each analyte / internal standard to a calibration curve constructed from extracted plasma. Mean extraction efficiency of fluoxetine and norfluoxetine respectively, was 92.3% and 87.1% compared to neat standards. The assay was linear from 10 – 500 ng/mL (>0.990) whereas

complex precision was less than 8.0% coefficient of variation at 50 and 500 ng/mL as determined from six measurements obtained over seven days.

Next, fluoxetine HCl was added to the Tang for the treated group while the control group continued to drink Tang only. The initial concentration of fluoxetine in Tang was appropriate to a total dose of 0.03 mg/kg in 50 ml. For a 10 kg monkey, that is 50 ml of 0.006 mg fluoxetine/ml Tang. As long as intake was stable, fluoxetine concentration was increased by 0.5 log unit (about three-fold) every three days until terminal concentration was reached such that monkeys consumed 2.0 mg/kg fluoxetine each day.

One-two weeks after terminal fluoxetine concentrations were reached, monkeys were placed in chairs 23 hours after consuming fluoxetine for measurement of trough blood levels. Since steady state blood levels of fluoxetine in human are achieved after 3-4 weeks (Lemberger et al., 1985), two additional blood samples were taken after this at 3-4 week intervals. Blood levels of fluoxetine were low in this initial period compared to the reported clinical range of 19-199 ng/ml of fluoxetine and 45-244 ng/ml of the active metabolite, norfluoxetine (Wilens et al., 2002). Therefore, after approximately two months exposure to 2.0 mg/kg, we increased the dose to 3.0 mg/kg/day in the treated group. After approximately one month at 3.0 mg/kg/day, we drew blood a several time points after fluoxetine (1, 2, 4 and 23 hours) on a monthly basis for three months. Based upon that information we concluded that peak fluoxetine levels, seen at two hours after administration, approximated clinically relevant levels 52-168 ng/ml (Blardi et al., 2002; Brunswick et al., 2002). Figure 2S illustrates blood levels of fluoxetine and norfluoxetine at trough after 3.0 mg/kg and sampled at 1, 2, 4 and 23 hours post-drug ingestion.

Drug exposure continued for a total of 39 weeks, with the final 30 weeks at 3.0 mg/kg/day. Monkeys were euthanized 20-24 hours after their last drug or vehicle exposure. For euthanasia, most

were initially sedated with the combination of midazolam (Versed, 0.3 mg/kg, i.m.) and medetomidine (Domitor, 0.06 mg/kg, i.m.). Three monkeys (two treated, one control) required additional sedation with Isoflurane. They were then administered a lethal overdose of pentobarbital (75 mg/kg, i.v.) via the saphenous vein. Immediately following euthanasia the cranial dome covering the brain was lifted using an autopsy saw (810 Autopsy Saw from Stryker, 810-2-11-REV, Kalamazoo, MI). The brain was bisected into hemispheres and each hemisphere dissected into coronal blocks, immediately frozen in chilled isopentane and frozen blocks stored at -80°C.

References

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Table S1. Rhesus monkey subjects characteristics

	Tattoo number	Weights(kg)	Age	Genotype
Control	97E058	8.9	10.3	LL
	92B521	11.6	15.6	LL
	95E030	8.55	12.3	LL
	RQ1791	9.9	12.7	LS
	95E111	8.8	12.3	LS
	RQ1798	8.1	12.7	LL
	mean ± SEM	9.31 ± 0.52	12.65 ± 0.7	
Treatment	RQ4032	9.75	10.1	SS
	94E028	8.8	13.5	LL
	95E024	8.8	12.3	LL
	97E013	6.75	10.4	LS
	AO816	8.4	15.7	LL
	RO1708	9.1	13.6	LL
	RQ1754	9.5	13.1	LL
	mean ± SEM	8.73 ± 0.37	12.67 ± 0.74	

Supplementary Figure Legends

Figure S1. Chromatogram of a 250 ng/mL extracted plasma standard illustrating the elution of the internal standard (citalopram), norfluoxetine, and fluoxetine at 3.56, 4.96, and 5.63 minutes respectively.

Figure S2. Graph of fluoxetine and norfluoxetine blood levels from monkeys. Blood levels were monitored monthly at trough, after 3.0 mg/kg and sampled at 1, 2, 4 and 23 hours post-drug ingestion. Data represent mean ± S.D.

Figure S3. Freud-1 protein levels by age at death of control subjects correlation.

Figure S4. Graph of control loading protein, β-actin levels in the prefrontal cortex of male and female MDD subjects and gender-matched controls.