Shrestha et al, "Fluoxetine Administered to Juvenile Monkeys: Effects on the Serotonin Transporter and Behavior"

Supplemental Methods

Rearing Conditions

Maternal separation was conducted under a standard protocol in which infants are separated from their mothers within 48 hours of birth and hand-reared by humans in a nursery for several weeks until they are able to feed themselves (typically four to six weeks) (1, 2). Maternally-separated monkeys were then transferred from the nursery and into group housing with three other like-reared, age-matched monkeys from the same birth year cohort. Maternally-separated monkeys remained in this condition until they were approximately six to eight months old, when they and all other infants from a given birth year (including those from both rearing conditions), were transferred to a large group housing condition.

In contrast, normally-reared monkeys spent the first six months of life living with their mothers and with other adults and infant pairs from the same birth cohort. Monkeys were housed in large indoor (2.5 x 3.0 x 2.5 m) / outdoor (2.5 x 3.0 x 2.5 m) runs that contained one to two males and six to eight females with their current year offspring. As noted above, between six and

eight months of age, these monkeys were transferred to a large pen with other maternally-separated and normally-reared monkeys from the same birth year.

At age two, monkeys were removed from group housing and pair-housed with a like-reared cage-mate. One member of each pair received fluoxetine and the other received placebo.

Social Behavior

Peer social behavior was evaluated in a series of round robin tests that took place between four and eight months after initiation of drug administration, and then again two to six months after drug cessation. Monkeys were removed from their home cage and placed in a larger "playcage" apparatus with another monkey from the same birth year cohort. Social testing was performed in a round robin format so that each monkey was tested four times, once with a different monkey from each rearing-drug group: 1) maternally separated with placebo administration, 2) maternally separated with fluoxetine administration, 3) normally reared with placebo administration, and 4) normally reared with fluoxetine administration. A mean behavioral score was then obtained for each monkey at each time point across all test pairs. The procedure occurred at two time points, once when monkeys were being treated with placebo or fluoxetine and a second time after treatment had discontinued. The same pairings among monkeys were retained across these two instances of the testing, that is during and after drug

administration. The frequency and duration of a number of behaviors from an adapted ethogram (3) that occurred in the first 15 min of each pairing were subsequently scored with Noldus 7.0 software by three experienced raters (EEN, BZ, JK). Behaviors from the ethogram were consolidated into nine composite measurements: locomotion, stereotypy, passivity, affiliation (physical proximity or grooming), dominance, submissiveness, coo vocalizations, bark vocalizations, and social (attack or bite) or nonsocial (cage shake) aggression. Intra- and inter-rater reliability were greater than 0.85 on all scored behaviors.

Round Robin Tests

Locomotion (state): Movement anywhere around the cage that does not include stereotypic movements. The movement must be continuous for more than one second.

Stereotypy (state): Repetitive and patterned motion throughout the cage (circling, rocking). Only score if the same pattern is repeated three times within a one-minute span and continue scoring for as long as the pattern or any part of it is repeated. Score each novel pattern separately.

Idiosyncrasy (point event): Repetitive or odd behaviors that fall under species-typical when not repeated, such as repetitive bouncing, or other repeated behaviors that may be incorporated into an individual animal's repertoire (i.e. head rolls, saluting, self-biting). An observed behavior

should only be coded as idiosyncrasy if there are three repetitions of the behavior within a minute, then code every time it occurs thereafter.

Passive (state): Sitting, standing, or hanging on one place away from other animals. The monkey must be in place for longer than one second.

Proximate (state) – focal send/receive: Passive behavior with distance to other animal of less than or equal to one half of cage quadrant length, or with other animal on same perch. The monkey must be passive for more than one second. Affiliation takes precedence over proximity.

Affiliation (state)—focal send/receive: Prosocial state behaviors including allogrooming, playing, huddling, clinging, or genital exploration.

Follow (state)—focal send/receive: Pursuing or being pursued by partner, either in affiliative or dominant manner. Must last for more than one second. Continue coding following until followee stops following. If direction of following switches, change modifier accordingly.

Dominance and submission (point event)—focal send (dominance)/receive (submission): May appear as displacement, crook tail, or chasing. In a full displacement where one animal sits or hangs in the place that the other occupied, code both focal send and receive accordingly.

Recipient must vacate spot within two seconds of sender's arrival. However, if the recipient moves from its spot when the other approaches, but the other does not occupy the spot, only code focal receive accordingly. Only code crook tail as focal send (never focal receive) when animal is holding the tail high in the air, clearly displaying to the other animal on the same level of the cage. Code chasing as dominance when there is a clear intention to show dominance from one animal to the other, almost as a moving displacement. May also code dominance as a point event if the behavior is continuous.

Attack (state)—focal send/receive: Vigorous biting and grasping, usually associated with screaming vocalizations, submissive posturing, and facial expressions. Also includes warning bites.

Aggression (state): Violent behavior not directed at other animal, usually in a bout of frustration. Examples include cage shake, vigorous running around cage, bouncing on all fours, or banging objects.

Fear (state): Crouching away from other animal or fear grimace, in which teeth are bared in a grin-like facial expression involving the retraction of the lips and clenched teeth.

Mount (point event)—focal send/receive: Mount onto rump of social partner; should include grasping and climbing onto partner's rear legs.

Positron Emission Tomography (PET) Scans

Both [^{11}C]DASB and [^{11}C](*R*)-RWAY PET radioligands were synthesized with high radiochemical purity (>99%), as previously described (4, 5). Before each scan, monkeys were immobilized with ketamine (10 mg/kg, i.m.) to allow endotracheal intubation. Monkeys were transported to the PET suite and placed under isoflurane anesthesia (1.5 - 2%) during the entire scan. The head was immobilized in a stereotactic frame, and vital signs were monitored throughout. To minimize the pharmacological effects of ketamine, scans started at least 120 min after ketamine administration.

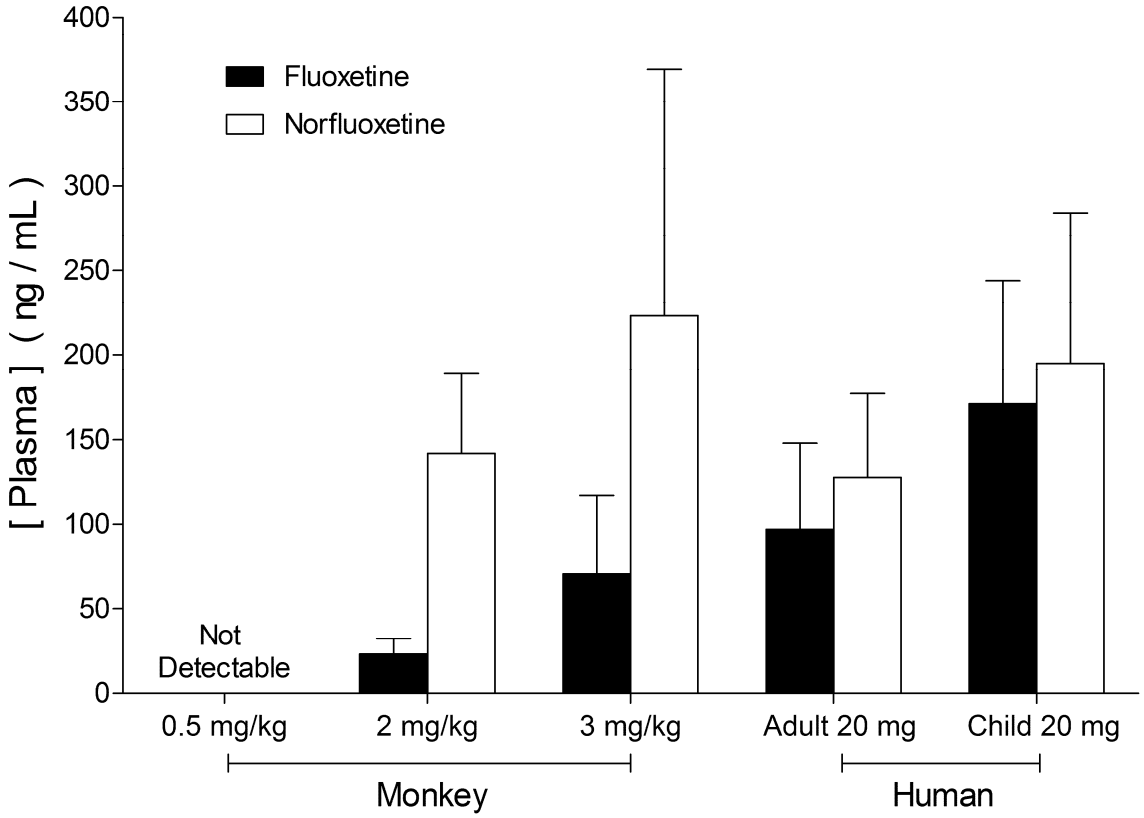
Prior to radioligand injection, a 10-min transmission scan was acquired using a ^{57}Co point source for attenuation correction. Each radioligand was injected as a bolus of 10 mL over one min via the saphenous or occasionally the cephalic vein. Images were reconstructed using 3D-filtered back-projection and had a resolution of 1.7 mm at full-width half-maximum. Scatter and attenuation corrections were applied. All monkeys also received a T1-weighted spoiled gradient

echo (SPGR) magnetic resonance imaging (MRI) scan with a human knee coil on a GE Signa 3T device (GE Healthcare).

Supplemental References

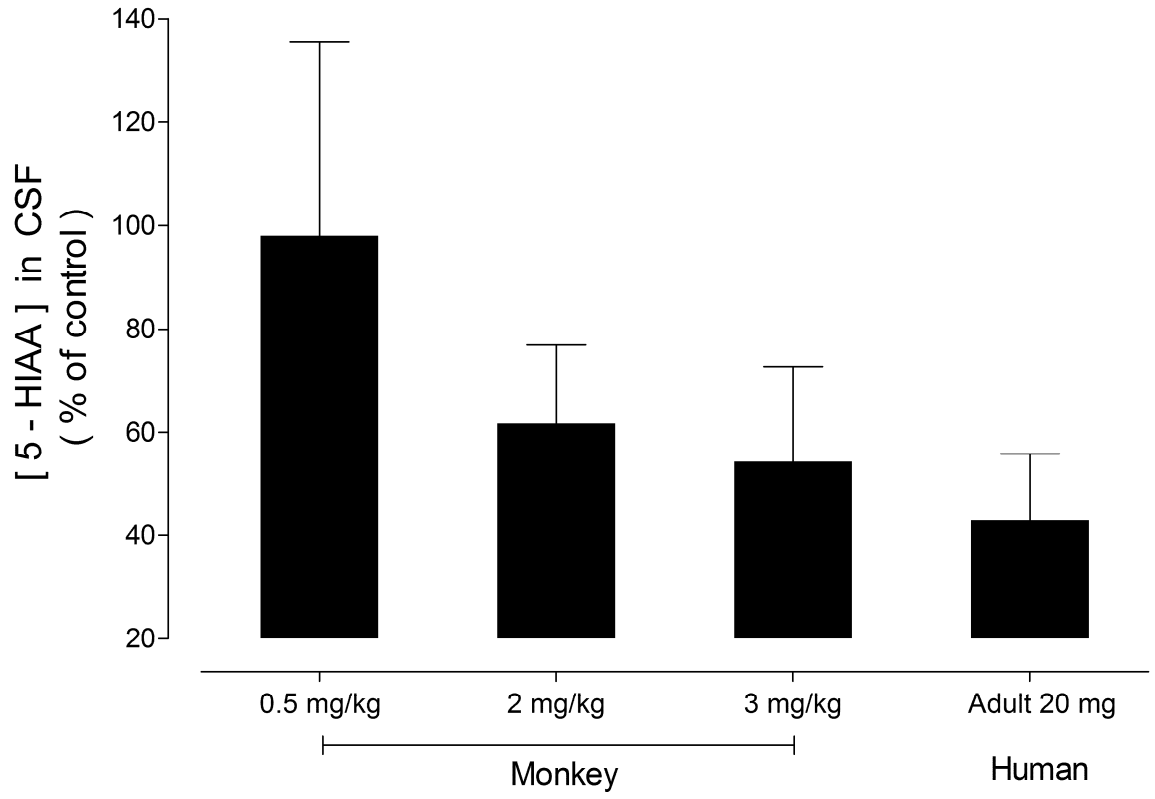
1. Shannon C, Champoux M, Suomi SJ. Rearing condition and plasma cortisol in rhesus monkey infants. Am J Primatol. 1998;46:311-21.
2. Nelson EE, Winslow JT. Non-human primates: model animals for developmental psychopathology. Neuropsychopharmacology. 2009;34:90-105.
3. Winslow JT, Noble PL, Lyons CK, Sterk SM, Insel TR. Rearing effects on cerebrospinal fluid oxytocin concentration and social buffering in rhesus monkeys. Neuropsychopharmacology. 2003;28:910-8.
4. Ichise M, Vines DC, Gura T, Anderson GM, Suomi SJ, Higley JD, Innis RB. Effects of early life stress on [^{11}C]DASB positron emission tomography imaging of serotonin transporters in adolescent peer- and mother-reared rhesus monkeys. J Neurosci. 2006;26:4638-43.
5. Yasuno F, Zoghbi SS, McCarron JA, Hong J, Ichise M, Brown AK, Gladding RL, Bacher JD, Pike VW, Innis RB. Quantification of serotonin 5-HT$_{1A}$ receptors in monkey brain with [^{11}C](R)-(-)-RWAY. Synapse. 2006;60:510-20.
6. Amsterdam JD, Fawcett J, Quitkin FM, Reimherr FW, Rosenbaum JF, Michelson D, Hornig-Rohan M, Beasley CM. Fluoxetine and norfluoxetine plasma concentrations in major depression: a multicenter study. Am J Psychiatry. 1997;154:963-9.
7. Wilens TE, Cohen L, Biederman J, Abrams A, Neft D, Faird N, Sinha V. Fluoxetine pharmacokinetics in pediatric patients. J Clin Psychopharmacol. 2002;22:568-75.
8. Sheline Y, Bardgett ME, Csernansky JG. Correlated reductions in cerebrospinal fluid 5-HIAA and MHPG concentrations after treatment with selective serotonin reuptake inhibitors. J Clin Psychopharmacol. 1997;17:11-4.

Figure S1



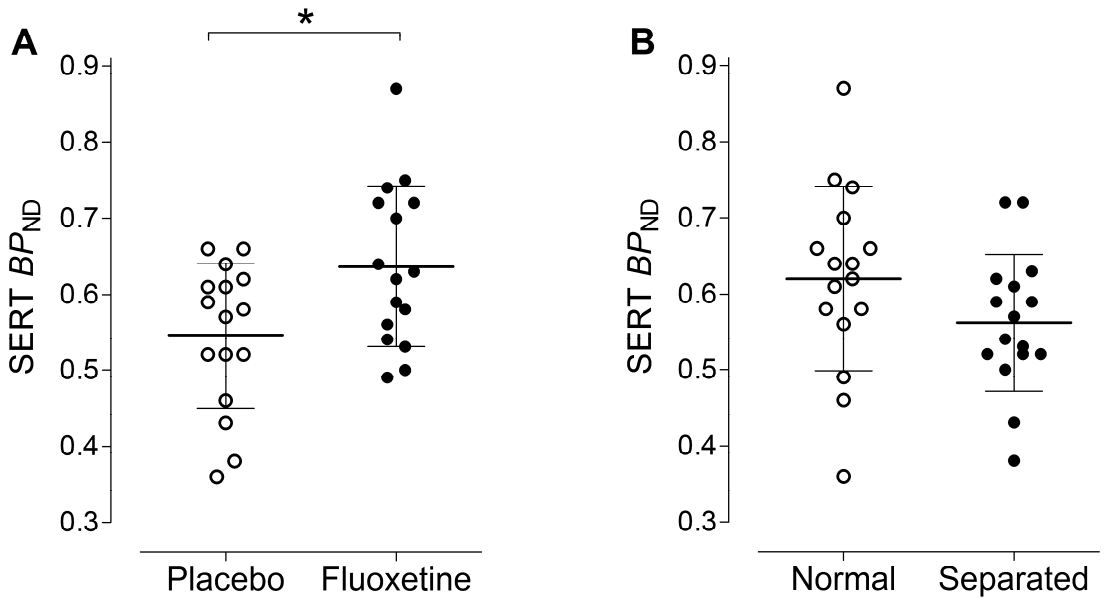
Supplemental Figure SF1. Plasma concentrations of fluoxetine and norfluoxetine in monkeys following four weeks of daily fluoxetine, administered orally at 0.5, 2.0, or 3.0 mg/kg. Plasma concentrations were measured in 6 to 12 monkeys for each of the three doses. For comparison purposes, plasma concentrations are provided for human adults (6) and children (7), both treated with 20 mg/day. Adults (n = 839) were 40 ± 11 years of age, and had been treated for approximately eight weeks. The children (n = 10) were 10 ± 1.9 years of age, and had been treated for approximately 8.5 weeks. Bars represent mean ± SD.

Figure S2



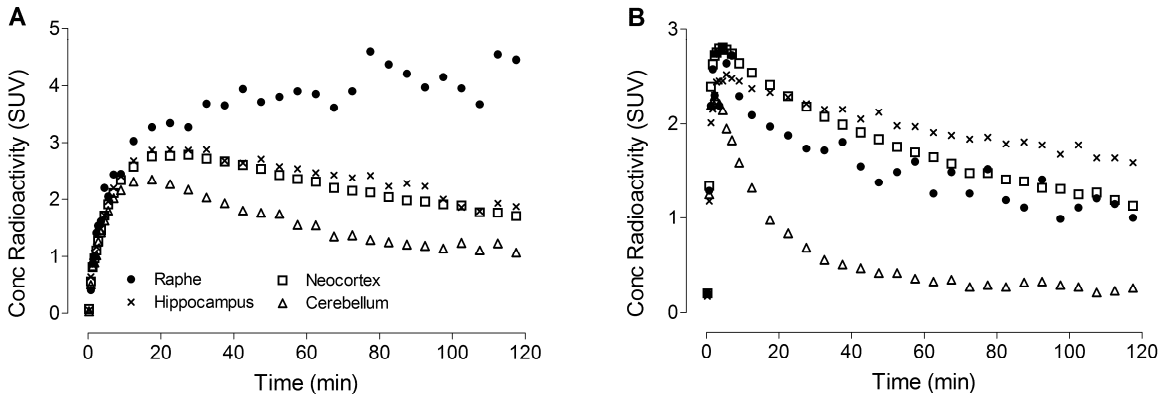
Supplemental Figure SF2. CSF concentrations of 5-HIAA expressed as percent of placebo-administered group following four weeks of daily fluoxetine orally administered at 0.5, 2.0, or 3.0 mg/kg. In humans (N=24), the mean concentration was 6.22 ± 2.06 ng/mL after approximately six weeks treatment with fluoxetine or fluvoxamine (8). Bars represent percent of control ± SD.

Figure S3



Supplemental Figure SF3. Effects of fluoxetine and maternal separation on SERT in hippocampus. A) Fluoxetine upregulated SERT by 17% (*p = 0.032 after correction for multiple regions). B) Maternal separation had no significant effect on SERT (p = 0.22). BP_{ND} – Binding potential; Bars represent mean ± SD.

Figure S4



Supplemental Figure SF4. Time-activity curves for [^{11}C]DASB and [^{11}C](*R*)-RWAY in raphe, hippocampus, neocortex, and cerebellum from a normally-reared monkey that received placebo. A) For [^{11}C]DASB, all regions except raphe had well-defined time of peak uptake (~20 min) and rate of washout during the 120-min scan. In this animal and in about ~20% of all animals, the concentration of radioactivity (standardized uptake value; SUV) in raphe continued to increase for the entire scan; B) For [^{11}C](*R*)-RWAY, all regions had a well-identified time of peak (~ 10 min) and rate of washout.

Supplemental Table ST1. Characteristics of rhesus monkeys and injected radioligands ([^{11}C]DASB and [^{11}C](*R*)-RWAY) in groups that received placebo or fluoxetine*

	Placebo n = 16 (mean ± SD)	Fluoxetine n = 16 (mean ± SD)
Rhesus monkeys		
Age (years)	4.69 ± 0.55	4.72 ± 0.56
Weight (kg)	7.41 ± 1.56	7.53 ± 1.48
[^{11}C]DASB		
Injected activity (MBq)	164 ± 33.6	158 ± 22.4
Specific activity (MBq/nmol)	80 ± 32.4	87 ± 54.8
Injected mass (nmol/kg)	0.34 ± 0.2	0.35 ± 0.20
[^{11}C](*R*)-RWAY		
Injected activity (MBq)	159 ± 34	156 ± 35.2
Specific activity (MBq/nmol)	100 ± 34	108 ± 43.2
Injected mass (nmol/kg)	0.25 ± 0.12	0.24 ± 0.16

*None of these variables differed significantly between monkeys who received placebo and those who received fluoxetine.

Supplemental Table ST2. Voxel-wise analysis showing the effects of fluoxetine in lateral temporal and posterior cingulate cortices.

Brain regions	Cluster size (voxels)	Cluster-level corrected *p* value	Voxel-level T-value*
Right lateral temporal	6217	< 0.001	5.64 5.02 4.87
Left lateral temporal	5480	< 0.001	5.60 5.27 4.78
Posterior cingulate: left and right	3547	0.001	4.85 4.34 4.29

Voxel volume = 0.51 mm^3 (0.8 mm x 0.8 mm x 0.8 mm)
*For each region, we report the three highest threshold values at the voxel level that survived the stringent family-wise error (FWE) statistical corrections.

Supplemental Table ST3. Effects of fluoxetine on behaviors measured 'during treatment' and 'after treatment' in normally-reared and maternally-separated monkeys.

Behaviors	Normally reared				Maternally separated				Significance
	Placebo (N = 8)		Fluoxetine (N = 8)		Placebo (N = 8)		Fluoxetine (N = 8)		
	During	After	During	After	During	After	During	After	
Duration									
Locomotion	272 ± *202*	203 ± *217*	236 ± *235*	228 ± *227*	217 ± *141*	160 ± *167*	152 ± *227*	144 ± *167*	# p<0.021*8=0.16 ***** p<0.048*8=0.38
Stereotypy	25 ± *114*	65 ± *255*	52 ± *157*	76 ± *152*	86 ± *180*	115 ± *273*	233 ± *440*	262 ± *414*	***** p<0.013*8=0.10
Passivity	196 ± *144*	295 ± *223*	208 ± *182*	260 ± *234*	200 ± *194*	267 ± *292*	126 ± *189*	158 ± *247*	# p<0.0002*8=0.002
Affiliative	99 ± *60*	26 ± *21*	96 ± *78*	29 ± *22*	87 ± *75*	48 ± *36*	82 ± *112*	27 ± *29*	# p<0.00006*8=0.0005
Frequency									
Dominance	4.0 ± *8.1*	3.3 ± *7.3*	2.0 ± *5.2*	1.2 ± *3.1*	3.3 ± *6.3*	3.0 ± *5.7*	1.4 ± *4.1*	1.6 ± *4.5*	****** p<0.038*8=0.30
Submissive	3.3 ± *7.5*	2.4 ± *5.9*	3.5 ± *5.6*	3.3 ± *5.7*	2.0 ± *5.8*	1.3 ± *2.4*	1.8 ± *4.8*	3.8 ± *5.2*	## p<0.049*8=0.39
Coo-Vocal	6.1 ± *13.8*	0.8 ± *2.4*	6.6 ± *26.6*	7.8 ± *37.3*	16.5 ± *52.4*	10.3 ± *37.3*	10.7 ± *34.6*	2.4 ± *4.4*	p>0.05*8>0.40
Bark-Vocal	0.6 ± *1.6*	1.2 ± *4.9*	4.5 ± *14.9*	4.2 ± *12.6*	7.0 ± *20.6*	18.7 ± *57.4*	12.6 ± *31.8*	4.8 ± *14.7*	***** p<0.033*8=0.26

The duration of the top four behaviors is expressed in seconds during a 10-minute observation period. The frequency of the bottom four behaviors is expressed as number of occurrences during the 10-minute observation period. Values are mean ± *SD*.Symbols denote the comparisons performed with repeated measures ANOVA: # main effect of period (i.e., before treatment vs. after treatment); ***** main effect of rearing; ****** main effect of drug (i.e., fluoxetine vs. placebo); and ## drug-by-period interaction.