Molecular fMRI of Serotonin Transport

Highlights
- Molecular imaging technology enables mapping of serotonin uptake dynamics
- Kinetic parameters of serotonin transport are obtained by compartmental modeling
- Serotonin transporter-dependent and -independent mechanisms are differentiated
- Inhibition of dopamine transporters blocks serotonin transport in some regions

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In Brief
Molecular-level MRI produces unprecedented three-dimensional profiles of serotonin clearance and its manipulation by drugs. Data indicate areas of peak clearance and quantify regional contributions of serotonin transporter-dependent and -independent mechanisms to neurotransmitter removal.

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Molecular fMRI of Serotonin Transport

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SUMMARY

Reuptake of neurotransmitters from the brain interstitium shapes chemical signaling processes and is disrupted in several pathologies. Serotonin reuptake in particular is important for mood regulation and is inhibited by first-line drugs for treatment of depression. Here we introduce a molecular-level fMRI technique for micron-scale mapping of serotonin transport in live animals. Intracranial injection of an MRI-detectable serotonin sensor complexed with serotonin, together with serial imaging and compartmental analysis, permits neurotransmitter transport to be quantified as serotonin dissociates from the probe. Application of this strategy to much of the striatum and surrounding areas reveals widespread nonsaturating serotonin removal with maximal rates in the lateral septum. The serotonin reuptake inhibitor fluoxetine selectively suppresses serotonin removal in septal subregions, whereas both fluoxetine and a dopamine transporter blocker depress reuptake in striatum. These results highlight promiscuous pharmacological influences on the serotonergic system and demonstrate the utility of molecular fMRI for characterization of neurochemical dynamics.

INTRODUCTION

Mechanisms of neurotransmitter transport within and around synapses govern the temporal characteristics and intensity of neural transmission (Amara and Kuhar, 1993; Kanner and Zomot, 2008; Masson et al., 1999). For several neurotransmitters, cellular reuptake following activity-dependent release is one of the most important determinants of signaling dynamics. Modulation of neurotransmitter reuptake therefore contributes to tuning neural network activity across the central nervous system (Castren, 2005). Potent addictive drugs such as cocaine act by inhibiting neurotransmitter reuptake transporters (Sulzter, 2011), and artificial manipulation of reuptake also provides a therapeutic strategy for the management of several psychiatric and neurological diseases (Iversen, 2000; Jensen et al., 2015). A particularly well-known example is the use of selective serotonin reuptake inhibitors (SSRIs) in the treatment of depression (Vaswani et al., 2003; Wong and Licinio, 2001). SSRIs competitively inhibit the serotonin transporter (SERT) and exert widespread effects ranging from reduction of pain and stress (Gorman and Kent, 1999) to induction of neurogenesis, plasticity, and learning (Chamberlain et al., 2006; Chen et al., 2011; Santarelli et al., 2003).

Experimental analysis of neurotransmitter kinetics in vivo is critical to understanding how these processes influence neural function on a regional or global level. Efforts to characterize neurotransmitter reuptake and redistribution patterns could also facilitate better understanding of neuropharmacological therapies (Kirsch et al., 2002, 2008) and might inform the development of improved treatments. Real-time measurements of serotonin (5HT), dopamine, and norepinephrine clearance in laboratory animals have been performed using chronamperometry and voltammetry (Bucher and Wightman, 2015), revealing some regional differences in uptake parameters and susceptibility to pharmacological perturbations (Callaghan et al., 2005; Park et al., 2010; Shu et al., 2014). Because these measurements sample only discrete points in the brain, however, they are ill suited to large-scale mapping of kinetic parameters. Radioligand imaging techniques such as positron emission tomography (PET) do provide spatially comprehensive information about tracer distribution, and have been used to measure SERT in vivo (Huang et al., 2010). With competition-based strategies, slowly varying neurotransmitter concentrations can sometimes be estimated (Laruelle, 2000; Paterson et al., 2013), but neurotransmitter transport rates based on these approaches have not been reported.

We previously introduced a family of imaging agents that enables mapping of extracellular neurotransmitter release using molecular-level fMRI (Brustad et al., 2012; Lee et al., 2014; Shapiro et al., 2010). The imaging probes are paramagnetic proteins that brighten T₁-weighted images in the absence, but not the presence, of bound monoamines, providing a means for quantifying concentrations of these neurotransmitters. Although the sensors must be invasively injected into the brain, their application in conjunction with MRI in animals permits molecular measurements over relatively large fields of view, with voxel volumes on the order of 0.1 µL and temporal resolution of seconds (Lee et al., 2014). For sensitivity reasons, our earlier in vivo studies were performed in the presence of neurotransmitter reuptake blockers, meaning that native monoamine reuptake rates could
not be reliably inferred from the imaging measurements. We speculated, however, that a different experimental strategy based on coinfusion of a sensor and its analyte could enable measurements of reuptake even for neurotransmitters like serotonin that rarely reach concentrations detectable by previous methods.

Here we put this idea to the test using a monoamine sensor called BM3h-2G9C6 (2G9C6), which displays a 0.7 μM dissociation constant for serotonin with more than 200-fold specificity compared with dopamine and norepinephrine (Brustad et al., 2012). We demonstrate that molecular imaging with this probe, in conjunction with compartmental modeling, enables three-dimensional mapping of serotonin transport in rat brains by fMRI. The method provides spatially resolved estimates of serotonin removal rates and their perturbation by monoamine transporter blockers, constituting a potentially powerful tool for in vivo analysis of neurochemistry and drug mechanisms.

RESULTS

An MRI Sensor Enables In Vivo Measurement of Serotonin Dynamics

The MRI sensor 2G9C6 is an engineered mutant of the 53 kD bacterial cytochrome heme domain P450-BM3h. The sensor’s potency as an MRI contrast agent is expressed by its $T_1$ relaxation ($r_1$). The 2G9C6 $r_1$ at 37°C and 9.4 T undergoes a 5-fold reduction upon serotonin (5HT) binding in vitro, dropping from 0.99 mM$^{-1}$ s$^{-1}$ to 0.19 mM$^{-1}$ s$^{-1}$ with addition of saturating 5HT (Figure S1A). A straightforward approach to imaging 5HT transport in vivo could be based on analysis of MRI signal following coinjection of the sensor with exogenous 5HT in rats, a strategy that parallels earlier point-measurement studies with electrochemistry (Daws et al., 1997). Our prediction was that gradual removal of 5HT bound to the sensor during and after infusion would increase the sensor’s mean relaxivity and produce a spatial profile of time-dependent, $T_1$-weighted MRI signal increases that depend on diffusion, convection, and localized reuptake in the brain; these parameters could then be quantified using kinetic modeling approaches. Monitoring transport using the 5HT-bound sensor coinjection procedure optimizes sensitivity to 5HT reuptake versus release, and it enables the full dynamic range of the sensor to be exploited while also resulting in continuously buffered 5HT concentrations within physiologically relevant, low micromolar levels.

To test our approach, we began by injecting 500 μM 2G9C6, either with or without equimolar 5HT, via paired cannulae implanted into the striatum of anesthetized rats (Figure 1A). The 2G9C6-5HT coinjection condition was predicted to yield maximal free 5HT concentrations of 18 μM in the infusion cannula, as determined by the $K_d$ of the sensor for 5HT. Infusions were performed over a 60 min period at a rate of 0.1 μL/min. MRI images were acquired continuously with 16 s per frame and in-plane resolution of 200 μm over six coronal 1 mm slices near the infusion cannula locations, both while the infusion was in progress and for a subsequent interval of 60 min. The injections themselves resulted in significant contrast enhancements over an approximately 3 mm diameter area (Figure 1B) that included regions of the caudate putamen (CPu), nucleus accumbens (Acb), lateral globus pallidus (LGP), lateral septum (LS), and bed nucleus of the stria terminalis (BST). To further analyze these enhancements, we defined rectangular regions of interest (ROIs) around each infusion site and examined the magnitude of MRI signal changes in areas that received clearly discernible doses of contrast agent—defined as those voxels that showed greater than 2% signal increase during the injections, corresponding to at least roughly 1 μM 5HT-bound sensor or 0.2 μM 5HT-unbound sensor. Among such voxels across five animals, an average image-signal enhancement of 19.4% ± 4.6% was observed during infusion of 2G9C6 in the absence of 5HT, whereas a lesser enhancement of 10.5% ± 1.4% was observed during coinfusion of the MRI sensor with 5HT. If the infused 2G9C6-5HT complex remained intact, without dissociation or removal of 5HT, the 5-fold lower $r_1$ of 2G9C6-5HT with respect to ligand-free 2G9C6 would have given rise to an approximately 5-fold lower signal change upon its injection. The fact that the 2G9C6-5HT signal change is only a factor of two lower therefore implies that close to 50% of the infused complex converts to the more MRI-visible 2G9C6, likely as a result of 5HT unbinding or removal during 2G9C6-5HT infusion.

During the postinfusion period, molecular fMRI time courses observed in the presence versus the absence of 5HT differed qualitatively. In the absence of coinjected 5HT, clear decreases in MRI signal were observed for most voxels near the cannula tip (Figure 1B, top). In contrast, in the presence of coinjected 5HT, minimal signal decreases and in some cases MRI signal increases were observed following the injection period (Figure 1B, bottom). These effects could be quantified at the level of single voxels (Figure 1C) and show that the extent of postinjection signal change varied systematically with position in both +5HT and −5HT infusion conditions. Similar findings were obtained in each individual experiment; the data could be compared in the form of MRI signal changes averaged over animals in rectangular ROIs defined as in Figure 1A. Results of this analysis (Figure 1D) indicated that the average signal change following injection of 2G9C6 without 5HT was a decrease of 4.3% ± 0.7%, whereas the change following coinfusion with 5HT was an increase of 1.5% ± 0.8%.

SERT Inhibition Alters Molecular fMRI Time Courses

The molecular imaging time courses of Figure 1D were consistent with our prediction that 5HT reuptake would lead to MRI signal increases in the sensor neurotransmitter coinjection approach, but we sought to establish the contribution of SERT activity more directly. To do so, we again performed the injection of 2G9C6 in the absence or presence of coinfused 5HT, but now we did so in a separate group of five animals that were pretreated by systemic injection of the SSRI fluoxetine (FLX, 5 mg/kg). The average MRI time course from animals that received 2G9C6-5HT coinjection after FLX (Figure 1E) in fact displayed little or no evidence of 5HT unbinding from the sensor, as would be expected if SERT-catalyzed removal of 5HT was reduced. Voxel-level time courses obtained in the presence of FLX (Figure S1B) were also similar to those observed during and after 2G9C6 injection without 5HT (Figure 1C, left). Quantification of the postinfusion MRI signal changes (Figure 1F) showed that an average signal decrease of 2.4% ± 0.4% was observed following coinjection
of 2G9C6 in the presence of 5HT and FLX. This decrease was significantly different from the increase seen under comparable conditions in the absence of FLX (t test, p = 0.013), and was not significantly different from the percent signal decrease observed following injection 2G9C6 in the absence of 5HT (p = 0.8). These results confirm that neurotransmitter reuptake...
contributes importantly to the MRI signal observed in the period following injection of 2G9C6 with 5HT.

FLX dependence of the molecular fMRI time courses could be resolved in individual brain regions. We examined image-signal dynamics in six anatomically defined ROIs corresponding to Acb, BST, CPU, LGP, and rostral and caudal subdivisions of LS (LSr and LSc, respectively; Figure 2A). MRI signals observed after infusion of 2G9C6-5HT in the presence or absence of FLX were compared for each ROI (Figure 2B). All regions except LSc showed substantially depressed MRI signal in the presence of FLX, consistent with inhibition of 5HT reuptake and its removal from the sensor. The largest responses to FLX were observed in Acb, CPU, and LSr. These data suggest that SERT-dependent 5HT transport parameters vary spatially and can be measured by MRI. The time courses are difficult to interpret rigorously in terms of 5HT unbinding from 2G9C6, however, because they reflect combined influences of diffusion, convection, and 5HT clearance over the course of the experiments.

Compartmental Modeling Yields 5HT Transport Parameter Estimates
In order to extract quantitative voxel-level 5HT transport parameters from the molecular fMRI data, we devised a simple compartmental modeling approach (Figure 3A). The model accounted for five microscopic processes: (1) infusion of 2G9C6-5HT complex at the cannula tips, modeled as a constant influx during the 60 min injection period; (2) transformation from 2G9C6-5HT to uncomplexed 2G9C6 without direct consideration of binding and unbinding of 5HT from the sensor. Omitting the kinetics of 5HT binding and unbinding from 2G9C6 was justified by stopped-flow measurements that revealed relatively short time constants for 5HT binding and unbinding from 2G9C6 (~40 s, Figure S3), compared with the much slower observed changes in 2G9C6-related MRI contrast within each voxel. At equimolar or excess sensor concentrations, 5HT levels would be restricted to low, buffered levels determined by the balance of 2G9C6-5HT and 2G9C6 in each voxel. The modeling approach was able to simulate individual voxel MRI signal time courses with high accuracy (Figure 3B). R² values averaged over voxels ranged from 0.85 to 0.95 for all animals; in each case, only voxels that showed minimal or negative signal change during contrast agent injection were excluded from the analysis. Comparison of observed and modeled image series also shows excellent agreement (Figure 3C), with both gross temporal features and spatial details reproduced in the best-fit simulation. The analysis procedure was applied to data obtained both in the absence and presence of FLX. In both cases, fitted convection constants ranged from 0 to 0.1 s⁻¹. The spatial distribution of convection constants was approximately centrosymmetric and similar across animals, indicating reproducibility of the experimental and modeling approaches.

5HT Removal Varies Spatially and Suggests Multiple Clearance Mechanisms
Maps of 5HT unbinding rates kU were obtained from the compartmental modeling procedure. Figure 4A displays kU values averaged across animals in both the −FLX and +FLX conditions (both n = 5). Data obtained in the absence of FLX show that substantial 5HT unbinding is apparent across much of the area for which data were obtained, and that particularly high 5HT removal is observed in a dorsomedial region including LS, BST, and parts of LGP and CPU. Values of kU are sharply reduced in animals pretreated with FLX, although some areas appear less affected than others. Average 5HT unbinding rates
were quantified in the six previously defined anatomical ROIs (Figure 4B). Consistent with the maps, mean \( k_U \) values were highest for the LS regions, where the observed unbinding rates were significantly higher than in Acb, CPu, and LGP (\( p \leq 0.037 \)). All ROIs except BST showed significant reduction of average \( k_U \) upon FLX treatment (\( p \leq 0.049 \)). Percent reduction in mean \( k_U \) varied from 40% ± 16% for LSc to 74% ± 8% for Acb. Both ROI and voxel-level model fitting results, therefore, reveal significant but varying effects of FLX on 5HT removal kinetics.

Kinetic modeling of the 2G9C6 molecular fMRI data also enables estimation of classical enzyme kinetic parameters (Figure 4C). By assuming a fast equilibrium between 2G9C6-5HT and 2G9C6 after the sensor infusion period, we determined effective free 5HT concentrations \( S_{eff} \), given by \( K_d[2G9C6-5HT]/[2G9C6] \). We also computed effective velocities of 5HT removal \( V_{eff} \), given by \( k_U[2G9C6-5HT] \). \( S_{eff} \) and \( V_{eff} \) values are plotted for each ROI in the presence and absence of FLX (Figure 4D). The range of \( S_{eff} \) values for each ROI results from time-dependent depletion of free 5HT during the postinfusion period. These estimated free 5HT concentrations range from 0 to 6 μM, consistent with the effects of sensor dilution and 5HT unbinding as the infused molecules spread throughout the brain during the imaging experiments. Corresponding 5HT removal velocities range from 0 to 20 nM/s. \( V_{eff} \) values in the \( 10^{-9} \text{ to } 10^{-8} \) M/s range are consistent with kinetic measurements from SERT-expressing cells (Blakely et al., 1991) and 5HT clearance rates recorded by in vivo voltammetry (Dankoski et al., 2014).

Several points about the graphs of \( S_{eff} \) versus \( V_{eff} \) are notable. First, \( S_{eff} \) values are systematically higher in the +FLX condition for all ROIs, showing that our molecular imaging and modeling methods successfully measure this expected result of SSRI treatment. Second, the graphs of \( S_{eff} \) versus \( V_{eff} \) in both –FLX

![Figure 3. Compartmental Modeling of Molecular fMRI Data](image-url)
and +FLX conditions are approximately linear ($R^2 > 0.84$), with only minimal evidence of asymptotic behavior even for the highest values of $S_{\text{eff}}$. Although these graphs are partially constrained by the kinetic model, the absence of maximal $V_{\text{eff}}$ values even for effective 5HT concentrations over 1 $\mu$M suggests that the dominant mechanisms of 5HT clearance do not saturate with increasing [5HT]. This adds to previous evidence that 5HT transporters behave more like nonsaturating channels (DeFelice and DeFelice, 1999) than like conventional saturating Michaelis-Menten enzymes at 5HT concentrations above the reported $K_m$ values of 0.1–0.3 $\mu$M (Blakely et al., 1991; Hagan et al., 2010). Finally, the FLX-dependent difference in slope for each ROI varies among regions, consistent with the calculated differences in $k_U$ (Figure 4B). Slopes of $S_{\text{eff}}$ versus $V_{\text{eff}}$ in principle indicate the rate constants for 5HT transport more directly than the $k_U$ values, and therefore provide further support for contributions of both FLX-inhibited and FLX-independent mechanisms to 5HT clearance in each region.

**Dopamine Transporters Contribute to Striatal Serotonin Removal**

A possible mechanism for FLX-independent 5HT removal from the brain is the nonspecific transport of 5HT by dopamine transporter (DAT) proteins. Electrochemical measurements and experiments in slices have shown that low micromolar 5HT concentrations can be taken up via DAT and packaged for re-release by dopaminergic neurons (Daws, 2009; Zhou et al., 2005). To assess the contribution of this route to the 5HT removal profiles obtained by molecular fMRI, we repeated the experiments of Figures 1–4 in five additional rats treated with 20 mg/kg of the DAT inhibitor GBR-12909 (GBR). Like FLX, GBR administration inhibited the relative increase of MRI signal observed following coinjection of 5HT with the 2G9C6 sensor into rat brain (Figure 5A), consistent with partial suppression of 5HT reuptake. Kinetic analysis of the data following methods of Figure 3 yielded a spatial map of 5HT removal rates in the presence of GBR (Figure 5B). The quality of fits ($R^2 \geq 0.85$) was comparable to those obtained without drugs or in the presence of FLX. The distribution of $k_U$ values appeared intermediate between those values observed in the absence of drugs and in the presence of FLX (Figure 4A).

Averaging the $k_U$ values over anatomically defined ROIs permitted quantitative comparison of the effects of GBR and FLX with respect to 5HT transport observed in the absence of drugs (Figure 5C). Whereas FLX-dependent suppression of 5HT removal was statistically significant ($p \leq 0.049$) in every ROI other than BST, GBR-dependent uptake suppression was significant only in Acb ($p = 0.03$), CPu ($p = 0.03$), and LSc

![Figure 4. 5HT Transport Parameters Obtained from Compartmental Modeling](image-url)
Figure 5. Perturbation of 5HT Transport by Inhibition of Dopamine Transporters

(A) Time series images similar to Figure 1B, showing sustained MRI signal increases following brain infusion of 2G9C6:5HT in the presence of the DAT blocker GBR.

(B) A map of 5HT unbinding rates observed after GBR pretreatment, color coded and labeled as in Figure 4A. Scale bar, 1 mm.

(C) Graph showing mean values of the 5HT removal rate-constant \( k_u \) observed for six ROIs in the presence of FLX or GBR, both shown relative to values observed in the absence of drug treatment.

(D) Effective free 5HT concentrations \( (S_{ew}) \) following infusion of 1:1 5HT:2G9C6 (top, high 5HT) or 1:2 5HT:2G9C6 (bottom, low 5HT) into rat brain, as estimated from compartmental model fitting. Scale bar, 1 mm.

(E) Mean 5HT unbinding rates \( (k_u) \) for six ROIs, determined by compartmental modeling of data obtained in the low and high 5HT regimens of (D), in the presence and absence of DAT inhibition with GBR. Suppression of \( k_u \) by GBR in CPu is significant under both high and low 5HT regimens \( (p = 0.03–0.05) \), while suppression of \( k_u \) in Acb and LSc is significant only at high 5HT \( (p = 0.05) \).

Data are represented as mean ± SEM.

\( p = 0.05 \). Interestingly, GBR suppressed 5HT removal in CPu to approximately the same extent as FLX did, by about 50%; this may reflect the particularly high concentration of DAT available for promiscuous 5HT uptake in this region \( (\text{Richfield, 1991}) \). In LSr, by contrast, where some of the greatest effects of FLX on 5HT removal were observed \((64\% ± 14\% \text{ suppression, } p = 0.002) \), GBR barely reduced \( k_u \) values at all \((13\% ± 18\% \text{ suppression, } p = 0.6) \); the mean \( k_u \) difference following FLX versus GBR treatment in LSr was significant \((p = 0.03) \).

In the experiments of Figures 5A–5C, free 5HT concentrations in the brain were estimated to reach maximal values of about 6 \( \mu M \) near the injection cannula, falling to submicromolar levels in areas further from the injection site \( (\text{Figure S4A}) \). 5HT concentrations above 1 \( \mu M \) are higher than reported stimulus-evoked 5HT concentrations in the brain \( (\text{Bunin and Wightman, 1998}) \); we wondered whether the apparent role of dopamine transporters in 5HT removal would persist if we adjusted the experimental conditions to maintain lower, uniformly submicromolar 5HT concentrations. We therefore applied our imaging and compartmental modeling approach following coinjection of 500 \( \mu M \) 2G9C6 with 250 \( \mu M \) 5HT, a 2:1 2G9C6:5HT mixture which is predicted to produce buffered free 5HT concentrations of at most 0.7 \( \mu M \). Raw MRI signal changes obtained following infusion of this low 5HT solution into rat striatum \( (\text{Figure S4B}) \) were similar to those shown in Figure 1C, with postinjection signal increases consistent with 5HT removal from 2G9C6 predominating over much of the ROI; as in Figure 1 and Figure S1B, signal increases were reversed in animals pretreated with the SERT inhibitor FLX, indicating the suppression of 5HT removal by the drug.

A group analysis of 2:1 2G9C6:5HT infusion experiments performed in the absence and presence of GBR \( (n = 5) \) was conducted. Results indicated that DAT inhibition with GBR continued to affect both raw molecular imaging trajectories and 5HT unbinding rate maps obtained from model fitting \( (R^2 \geq 0.84) \), even though free 5HT \( (S_{ew}) \) levels were observed to remain below 600 nM—about 10-fold lower than peak 5HT concentrations following equimolar 2G9C6:5HT infusion \( (\text{Figures 5D and S5}) \). Comparison of ROI-averaged \( k_u \) values \( (\text{Figure 5E}) \) showed a trend toward higher \( k_u \) values in experiments where higher [5HT] was present \( (\text{Figure 5E}) \), although the differences were not statistically significant for individual ROIs \( t \text{ test, } p > 0.06 \). Similar results were observed in the presence of FLX \( (\text{Figure S6}) \). Most ROIs showed a GBR-dependent reduction in mean 5HT unbinding rates in both low and high 5HT conditions,
on average by 29% and 34%, respectively. Importantly, the $k_u$ reductions in CPu were statistically significant in both experimental regimens ($p \leq 0.05$), suggesting that DAT-mediated 5HT transport remains a factor over a wide range of 5HT concentrations. In contrast, the GBR-induced $k_u$ reductions in Acb and LSc observed at high 5HT levels were not significant at low 5HT. In each ROI, GBR-dependent $k_u$ decreases were less than or equal to those observed in the presence of FLX (Figure S6D), but the differences between the two drugs were not statistically significant.

**DISCUSSION**

This work demonstrates that three-dimensional functional imaging of neurotransmitter transport can be performed in vivo using the MRI-detectable 5HT sensor 2G9C6. The approach involves coinfusing exogenous 5HT initially in complex with 2G9C6 into the brain and then analyzing fMRI signal changes that result from neurotransmitter dissociation from the sensor. A customized multi-voxel compartmental modeling approach is used to extract transport parameters from the raw MRI data and to calculate the rate of 5HT removal at each point, notwithstanding the inhomogeneous distributions of both neurotransmitter and MRI sensor. The effects of drugs such as the antidepressant FLX and the DAT blocker GBR can be mapped and compared.

During intracranial infusion of the 5HT-2G9C6 mixture, convective forces allow the sensor to spread over volumes of tens of microliters, permitting kinetic measurements over brain regions far more expansive than those accessible by point-measurement techniques like electrochemistry and microdialysis. In principle, the coverage of the molecular fMRI technique could be further extended by performing multiple injections in parallel, or by using minimally invasive trans-blood-brain barrier delivery techniques (Levyveld et al., 2010). Although the brain coverage might still be limited compared with radioligand-based neurotransmitter measurements, these other methods cannot distinguish between intracellular and extracellular ligands, and are therefore inherently ill suited to monitoring microscopic intravoxel transport processes such as 5HT reuptake. In contrast, our approach could monitor the process of 5HT removal from its binding equilibrium with the MRI sensor 2G9C6 within each voxel of the tissue, reflecting combined contributions from both extrasynaptic and synaptic regions accessible to the sensor because of its small diameter (~6 nm).

The fact that we did not include unbound 5HT in our modeling approach is a possible source of error, but the decision to treat 5HT only via its interactions with 2G9C6 was required for computational efficiency and justified by the relatively low concentrations of free 5HT in equilibrium with the neurotransmitter bound to the sensor. Because of the limited sensitivity afforded by 2G9C6, our molecular fMRI experiments involved intracranial injection of relatively concentrated solutions including total [5HT] up to 500 μM. Although our procedure introduced high total amounts of 5HT into the injected regions, the concentration of free 5HT—the unbound neurotransmitter level actually “seen” by transporters and other interaction partners—could be experimentally set by manipulating the ratio of 2G9C6 and 5HT infused into the brain. Using this approach, we performed separate sets of experiments that resulted in free 5HT concentrations of approximately 0–6 μM or 0–0.6 μM in the brain, and we observed largely similar transport constants and susceptibility to pharmacological perturbation in the two regimens.

The most immediate outcome of our study is the determination of unprecedented volumetric maps of 5HT transport parameters and their perturbation by FLX and GBR. Such data were not accessible to previous techniques and required development of the molecular imaging and compartmental modeling paradigm we introduce here. Unlike radioligand or immunohistochemical studies of SERT and DAT localization, the molecular fMRI data we present directly reflect 5HT dynamics themselves, as detected via binding of 5HT to the 2G9C6 MRI sensor. For this reason, the sensor unbinding rates we determined ($k_u$ values) could be affected by multiple processes including, but not limited to, SERT- or DAT-mediated cell uptake of 5HT. Additional factors include 5HT removal mediated by other transporters (Daws et al., 1998; Eiden et al., 2004; Eiden and Weihe, 2011) or bulk transport mechanisms (Ilfiff et al., 2012), sequestration of 5HT by specific or nonspecific cell binding, leakage of interstitial 5HT into the bloodstream (particularly near cannula implantation sites), and destruction of 5HT via cell-independent mechanisms such as spontaneous oxidation (Wegener et al., 2000). With the exception of cannula-related injury, all of these factors are relevant to normal brain biology.

Several observations suggest that SERT activity was a major contributor to 5HT sensor unbinding rates. First, both raw post-infusion MRI signal (Figure 2) and $k_u$ rates (Figure 4) were suppressed in each ROI by application of the SSRI FLX. 5HT removal rates were reduced by an average of over 50% among all ROIs in the presence of FLX, and were statistically significant in all regions except BST. Second, quantitative interpretation of the molecular fMRI data in terms of free 5HT transport velocities (Figure 4D) produced results comparable to previously reported transport rates and supports a previously proposed mode of nonsaturating SERT activity dependence on 5HT concentration (DeFelice and Goswami, 2007; Petersen and DeFelice, 1999). Spatial features of the MRI-based 5HT transport maps reported in Figure 4 may also suggest a dominant role for SERT. In particular, the peak $k_u$ values we discovered in regions of LS are consistent with earlier autoradiographic results indicating higher SERT expression in LS compared with CPu and surrounding structures (De Souza and Kuyatt, 1987; Jupp et al., 2013), as well as with microdialysis measurements showing greater effects of FLX on extracellular 5HT concentrations in LS than in striatum (Kirby and Lucki, 1997). LS subregions also displayed relatively high $k_u$ in the presence of FLX, as well as suppression of $k_u$ values by the DAT inhibitor GBR, suggesting that SERT-independent 5HT removal mechanisms may be especially active in this region.

A striking result of the experiments is the finding that the dopamine transport inhibitor GBR substantially perturbs 5HT removal in some of the brain areas examined, a likely consequence of promiscuous DAT activity (Daws, 2009) which can now be measured and mapped in vivo by our imaging method. Our observation of DAT-dependent 5HT transport in regions of the basal ganglia is consistent with earlier studies documenting...
GBR-mediated reduction of 5HT uptake in striatum (Callaghan et al., 2005; Shu et al., 2014) as well as with evidence that striatal concentrations of 5HT and 5HT metabolites are increased by selective DAT inhibition in normal or SERT knockout animals (Shen et al., 2004; Wong et al., 1993). In our molecular fMRI studies, GBR-mediated k_{u} suppression was most pronounced in the CPUs, an area known for high DAT expression (Ciliax et al., 1995; Richfield, 1991), where inhibition of 5HT removal was consistently observed in the presence of free 5HT concentrations that peaked from 0.6 to 6 μM. In the presence of micromolar concentrations of free 5HT, but not nanomolar levels, GBR also significantly depressed 5HT uptake in Acb. The difference between Acb and CPUs may arise in part from the fact that Acb displays lower DAT and higher SERT expression than CPUs (De Souza and Kuyatt, 1987; Wilson et al., 1994); this expression pattern could also explain the comparatively greater effect of FLX on k_{u} values in Acb than CPUs (Figure 5C).

The sensitivity of 5HT transport to both FLX and GBR in striatal areas contrasts with the more selective effects of FLX in LSr, which were particularly notable at higher neurotransmitter concentrations. LSr exhibits extensive serotonergic projections from dorsal raphe (Waselus et al., 2011) and also expresses particularly high levels of 5HT_{1A} receptor (Pazos and Palacios, 1985), the 5HT receptor subtype most directly implicated in the clinical efficacy of SSRIs antidepressant drugs (Celada et al., 2013). Activation of these receptors appears to produce anxiolytic effects (Singewald et al., 2011). Although the mechanisms by which SSRIs affect the serotonergic system are complex (Stahl, 1998; Walker, 2013), it is possible that the relative potency of FLX in subregions of LS is specifically important for its antidepressant effects. This idea could be further developed in part by application of the 5HT molecular imaging methodology in conjunction with additional drugs or manipulations. More generally, the molecular fMRI paradigm we introduce here provides a unique capability for spatial and temporal analysis of drug action on neurochemical processes in the brain and could aid importantly in the characterization or selection of substances destined for clinical use.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**

The hexahistidine-tagged cytochrome P450 BM3 heme domain variant BM3h-2G9C6 (2G9C6) was prepared following previously described procedures (Brustad et al., 2012; Lee et al., 2014; Shaprio et al., 2010). Briefly, overnight cultures of E. coli BL21 (DE3) cells carrying a pCWiOr(+)-plasmid encoding 2G9C6 regulated by a tandem Ptac promoter were inoculated at a 1:100 volumetric ratio into terrific broth (TB) medium containing carbenicillin at 100 μg/mL. Cultures were grown at 37°C to an optical density of 600 nm in the 0.8–1.0 range, and expression was induced by addition of 0.6 mM isopropl-β-D-thiogalactopyranoside for 12–16 hr at 30°C in the presence of 0.6 mM 5-amineolevulininate. Harvested cell pellets were lysed in BugBuster (EMD Millipore) with lysozyme and benzonase according to the manufacturer’s protocol, and a protease inhibitor cocktail (Set III EDTA-free; EMD Millipore) was added at 1:100 dilution. 2G9C6 was affinity purified from centrifugally clarified lysate using Ni-NTA agarose resin (QIAGEN), concentrated in a 30 kDa molecular weight cutoff ultracentrifugation device (Amicon Ultra, EMD Millipore), further purified by anion exchange chromatography (HiTrap Q XL, GE Healthcare BioSciences), and exchanged into phosphate buffered saline (PBS [pH 7.4]) using a desalting column (PD10, GE Healthcare Biosciences). Aliquots of purified protein were flash frozen in liquid nitrogen and stored at −80°C. 2G9C6 concentrations were determined by quantifying CO binding by the reduced protein using a molar extinction coefficient of 91 mM−1 cm−1 at 450 nm.

**Relaxivity Measurement**

Longitudinal T_{1} relaxivity (r_{1}) of purified 2G9C6 was quantified in a 9.4 T Avance II MRI scanner (Bruker Instruments) at 37°C. One-half of a 384-well microtiter plate was loaded with protein solutions with a range of concentrations in PBS (pH 7.4) with or without saturating serotonin (5HT, 2 mM). Temperature of the sample was maintained in the scanner bore using a circulating water bath and monitored using an MRI-compatible temperature probe during the scan. A series of T_{1}-weighted scans of a 2 mm slice across the plate were acquired using a spin echo-pulse sequence with echo time (TE) of 10 ms and recycle times (TR) of 116–3,125 ms. Intensity values in square ROIs centered on each plate well were determined from reconstructed magnitude images and fit to exponential decay curves to determine relaxation rates (R_{1} values) for each well using custom routines written in MATLAB (Mathworks). The slopes of R_{1} versus protein concentration were evaluated to determine r_{1} values.

**Stopped-Flow Analysis of Protein-Ligand Binding Kinetics**

Kinetics of 5HT binding and unbinding to BM3h-2G9C6 in vitro were studied using an Applied Photophysics DX-18MV SF stopped-flow spectrophotometry system operating at room temperature. Protein and 5HT solutions formulated in PBS were mixed in 1:1 volume ratio to final concentrations of 2.5 μM 2G9C6 and 20, 30, 40, or 60 μM 5HT. First-order rate constants were determined by exponential fitting to absorbance traces measured at 415 nm, with four or more replicates performed per condition. Forward and reverse rate constants were determined by linear fitting to these data to obtain values of k_{on} = 3.2 × 10^{10} M^{−1} s^{−1} and K_{D} = 0.023 s^{−1}. Data analysis was performed in MATLAB.

**Animal Use**

Male Sprague-Dawley rats (250-300 g) were purchased from Charles River Laboratories and used for all in vivo experiments. Animals were housed and maintained on a 12 hr light and dark cycle and permitted ad libitum access to food and water in strict compliance with the Committee on Animal Care (CAC) guidelines of the Massachusetts Institute of Technology.

**Preparation for Intracranial Injection and Imaging**

Intracerebral guide cannulae were implanted surgically to facilitate intracranial injection of 2G9C6 in MRI experiments. Each animal was anesthetized with isoflurane (4% induction, 2% maintenance), shaved, and mounted on a rodent stereotoxic device (Kopf Instruments) with heating pad. Heart rate and blood oxygenation were continuously monitored using a pulse oximeter (Nonin Medical) during all subsequent procedures. The scalp was retracted and small holes were drilled into the skull above the target sites. Two MRI-compatible 2 mm long guide cannulae (22 gauge; PlasticsOne) were implanted approximately 1.2 mm anterior and 1.5 mm lateral to bregma. A custom-fabricated plastic headpost was placed in front of the guide cannulae. Dental cement was applied to secure the implants rigidly in place. Each animal recovered from anesthesia under supervision. Buprenorphin (0.05 mg/kg) was injected subcutaneously after surgery and then twice daily over a 3-day monitoring period.

Immediately prior to contrast agent injection, MRI-compatible injection cannulae were mounted below a pedestal, PlasticsOne were connected to microtubing prefilled with 15 μL of 500 μM 2G9C6 with 0, 250, or 500 μM 5HT, resulting in predicted free 5HT concentrations of up to 0, 0.7, and 18 μM, respectively, as determined by 2G9C6-dependent buffering with the reported K_{D} of 0.7 μM (Brustad et al., 2013). Injection mixtures were all formulated in PBS. The injection cannulae were slowly lowered into the previously implanted guide cannulae while infusing contrast agent at a small injection rate to prevent air from becoming trapped during insertion. Injection cannulae were secured to the guide cannula with dental cement, and contrast agent injection was paused. The animal was then transferred to a plexiglass cradle covered with a water heating blanket to maintain body temperature and inserted into a transmission-only volume coil (Bruker Instruments) in conjunction with a receive-only surface coil (Doty Scientific) mounted on the head of the animal.
The animal was positioned at the isocenter of a 9.4 T Bruker Avance II scanner. The heart rate, spO2 level, and expired CO2 levels were monitored throughout the scan using Nonin 8600P pulse oximeter (Nonin Medical). Heart rate was maintained at 360–380 bpm. The animal was maintained with continuous delivery of 1.5% isoflurane for the duration of the scanning session. Some animals were pretreated by intraperitoneal injection of the SSRI fluoxetine hydrochloride (FLX, 5 mg/kg) or the dopamine transporter blocker GBR-12909 (GBR, 20 mg/kg). Imaging and 2G9C6 infusion commenced approximately 90 min after drug pretreatment in these cases.

MRI Data Acquisition
Anatomical scans, T1, and T2-weighted scan series were obtained from each animal. Multislice anatomical images with 200 μm in-plane resolution over six 1 mm coronal slices were obtained using a gradient echo-pulse sequence with a repetition time (TR) of 250 ms, echo time (TE) of 15.7 ms, field of view (FOV) of 25.6 x 12.8 mm, data matrix of 128 x 64 points, four averages, and 64 s total scan time. T1 maps were computed using data from a rapid acquisition with refocused echoes (RARE) pulse sequence with eight TR values (321, 600, 900, 1,000, 1,500, 2,000, 2,500, and 3,000 ms), TE of 10 ms, RARE factor of 4, FOV of 25.6 x 12.8 mm, data matrix of 64 x 32 points, and six 1 mm coronal slices with four averages and 378 s total scan time. Scan series for functional imaging were obtained using a gradient echo-pulse sequence with a flip angle of 30°, TE of 250 ms, TR of 250 ms, FOV of 25.6 x 12.8 mm, data matrix of 128 x 64 points, and 1 mm coronal slice thickness, with 16 s scan time per image. Scans were obtained consecutively for 120 min; during the first 60 min only, 2G9C6 solutions were infused at a constant rate of 0.12 μL/min.

Histology and Confocal Imaging
Following MRI experiments described above, rats were placed under terminal anesthesia using 5% isoflurane and transcendally perfused with phosphate buffer containing heparin (Hospira) and then with 4% w/v paraformaldehyde (Sigma-Aldrich). Brains were then removed and left overnight in 4% parafomaldehyde, followed by a wash in phosphate buffer saline. Coronal sections of 30 μm thickness were cut using a vibratome (VT1200, Leica Biosystems) across a range extending ~3 mm anterior and posterior from the injection cannula site. Antibody staining for SHT transporter was done using standard procedures at a dilution of 1:200 of primary antibody (anti-HTT/SERT, Frontier Institute Co.). Confocal imaging was performed using Nikon A1R Ultra-Fast Spectral Scanning Confocal Microscope.

Compartmental Modeling
Postprocessing and model fitting to the data were performed using customized routines in MATLAB and C. Raw image time series were temporally low-pass filtered (<0.1 Hz), and time courses from voxels in a 4.8 x 4.8 x 3 mm ROI near the 2G9C6 infusion sites were obtained.

These time courses were then jointly fitted to a compartmental model. The model describes the transfer and evolution of both 5HT-bound and 5HT-unbound 2G9C6 according to four processes: (1) Diffusion of bound and unbound sensor between adjacent nondiagonal voxels was modeled using a single first-order rate constant $k_{D}$. (2) Convective transfer of bound and unbound 2G9C6 to adjacent nondiagonal voxels was modeled using voxel-specific first-order rate constants $k_{Cijk}$ (i,j, and k are voxel indices). (3) Endocytosis and trapping of bound or unbound sensor was modeled using voxel-specific rate constants $k_{Tijk}$. (4) Removal of mHT resulting in net conversion of SHT-bound to unbound 2G9C6 was modeled using voxel-specific first-order rate constants $k_{Rijk}$.

$t_{i,j,k}$ values observed for each voxel at time $t$ were therefore described by the following expressions,

$$ R_{tijk}(t) = R_{i,j,k}(t+1) - B_{i,j,k}(t) - U_{i,j,k}(t) + R_{i,j,k}(t) + R_{i,j,k}(t) + R_{i,j,k}(t) + R_{i,j,k}(t), $$

where $R_{i,j,k}$ is the average background $R_{i,j,k}$ value observed in un.injected brain, determined to be 0.24 s⁻¹. Amounts of the bound and unbound 2G9C6 sensor $B_{i,j,k}(t)$ and $U_{i,j,k}(t)$, as well as corresponding trapped species $B_{i,j,k}(t)$ and $U_{i,j,k}(t)$, were updated for each time step $t$ to $t+1$, according to the following rules:

$$ B_{i,j,k}(t+1) = B_{i,j,k}(t) - (4 + 2V)(k_{b} + 2k_{i,j,k})B_{i,j,k}(t) - k_{i,j,k}B_{i,j,k}(t) + k_{0} + k_{Cijk}B_{i,j,k}(t) + k_{0} + k_{Cijk}B_{i,j,k}(t) + (k_{0} + k_{Cijk}B_{i,j,k}(t) + (k_{0} + k_{Cijk}B_{i,j,k}(t) + V(k_{0} + k_{Cijk}B_{i,j,k}(t) + V(k_{0} + k_{Cijk}B_{i,j,k}(t) - k_{i,j,k}B_{i,j,k}(t) $$

$$ U_{i,j,k}(t+1) = U_{i,j,k}(t) - (4 + 2V)(k_{b} + 2k_{i,j,k})U_{i,j,k}(t) + k_{i,j,k}U_{i,j,k}(t) + k_{0} + k_{Cijk}U_{i,j,k}(t) + k_{0} + k_{Cijk}U_{i,j,k}(t) + (k_{0} + k_{Cijk}U_{i,j,k}(t) + (k_{0} + k_{Cijk}U_{i,j,k}(t) + V(k_{0} + k_{Cijk}U_{i,j,k}(t) + V(k_{0} + k_{Cijk}U_{i,j,k}(t) - k_{i,j,k}U_{i,j,k}(t) $$

$V$ is a parameter that adjusts diffusion and convection rates to account for the longer voxel dimension in the MRI slice direction. Setting this parameter to 0.075 resulted in a physically justified equivalent spread of contrast agent between versus within slices. $k_{Cijk}$ values were set to zero for the second half of each time series, when contrast agent infusion had stopped. The only model parameter which was arbitrarily fixed was $k_{D}$, the diffusion rate constant; this parameter was set to 0.0004 s⁻¹, which resulted in diffusion equivalent to that observed for macromolecules similar in size to 2G9C6 according to previously published biophysical measurements (Syková and Nicholson, 2008; Tao and Nicholson, 1996). Fixing $k_{D}$ limited the potential for overfitting or unphysical refinement of this parameter during model fitting.

Parameter optimization was performed using MATLAB’s internal least-squares fit routines. Voxels included were those exhibiting positive signal changes throughout the 60 min of injection. The input data to the fitting algorithm consisted of a 24 x 24 x 3 matrix of MRI signal intensities in three adjacent image slices, sampled from the low-pass filtered experimental data at 11 equally spaced time points over the 120 min long injection and postinjection periods. The output consisted of best fit values for parameters $k_{C}, k_{b},$ and $k_{T}$, as well as corresponding time trajectories calculated using these values. Function tolerance for the termination of the optimization algorithm was set to 10⁻⁷. All refined models fit the data with average $R^{2}$ values ranging from 0.84 to 0.97 in each animal. To ensure that the literature-based choice of $k_{D}$ did not substantially bias the fitting results, we varied $k_{D}$ while recomputing the goodness of fit for representative datasets obtained under no drug or +FLX conditions. In these tests, we artificially chose diffusion rate constants from across a 10-fold range which exceeds any natural variability likely to be found in brain tissue. We found that disparate $k_{D}$ values only slightly compromised the goodness of fit, from an average $R^{2}$ of 0.90 to an average of 0.87. This demonstrates that the precise value of the diffusion parameter has minimal effect on the performance of our modeling.

Mean $k_{D}$ maps (Figures 4A, 5B, SS, and SE) were generated by averaging model data from individual animals among voxels for which data from two or more animals were available. An analogous procedure was used to compute the free SHT concentration maps in Figures 5D, SS, and SS. Average ROI values were computed by first averaging the ROI signal for a given dataset and then averaging across datasets from each animal. Effective free SHT concentrations ($S_{a}$) and SHT removal velocities ($V_{a}$) were computed using the formula $S_{a} = K_{D}[2G9C6-SHT]/[2G9C6]$ and $V_{a} = k_{D}[2G9C6-SHT]$. Statistical tests were performed using MATLAB. All error bars and error margins noted in the text denote SEM of multiple measurements unless otherwise noted.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.09.048.

AUTHOR CONTRIBUTIONS
A.H. performed the molecular fMRI experiments. A.H. and A.J. designed the research, analyzed the data, and wrote the paper. L.X.C. and T.L. participated.

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REFERENCES


