



Voltammetric evidence for discrete serotonin circuits, linked to specific reuptake domains, in the mouse medial prefrontal cortex

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ARTICLE INFO

Keywords:

6 max.) FSCV

Carbon fiber microelectrodes

Michaelis-menten

Uptake 1

Uptake 2

MFB

ABSTRACT

The medial prefrontal cortex (mPFC) is an important brain region, that controls a variety of behavioral and functional outputs. As an important step in characterizing mPFC functionality, in this paper we focus on chemically defining serotonin transmission in this area. We apply cutting-edge analytical methods, fast-scan cyclic voltammetry (FSCV) and fast-scan controlled adsorption cyclic voltammetry (FSCAV), pioneered in our laboratory, for the first real-time *in vivo* analysis of serotonin in the mPFC. In prior *in vivo* work in the substantia nigra, pars reticulata, we found that our sub-second measurements of a single evoked serotonin release were subject to two clearance mechanisms. These mechanisms were readily modeled via Uptake 1, mediated by the serotonin transporters (SERTs), and Uptake 2, mediated by monoamine transporters (dopamine transporters (DATs), norepinephrine transporters (NETs), and organic cation transporters (OCTs)). Here in the mPFC, for the first time to our knowledge, we observe two release events in response to a single stimulation of the medial forebrain bundle (MFB). Of particular note is that each response is tied to a discrete reuptake profile comprising both Uptake 1 and 2. We hypothesize that two distinct populations of serotonin axons traverse the MFB and terminate in different domains with specific reuptake profiles. We test and confirm this hypothesis using a multifaceted pharmacological, histological and mathematical approach. We thus present evidence for a highly elaborate biochemical organization that regulates serotonin chemistry in the mPFC. This knowledge provides a solid foundation on which to base future studies of the involvement of the mPFC in brain function and behavior.

1. Introduction

The medial prefrontal cortex (mPFC) has garnered substantial interest in recent times amongst a diverse group of experts (Calipari et al., 2018; Guise and Shapiro, 2017; Kim et al., 2017). Cutting-edge research has centered on the role of the mPFC in differentiating memories (Guise and Shapiro, 2017) and suppressing reward-seeking behavior (Kim et al., 2017) via projections to other brain regions. Recent work has identified a key factor in the mPFC which may be imperative to treating addiction without the potential for abuse (Calipari et al., 2018). These studies, among others, highlight the many functions of the mPFC and the importance they play in a variety of contexts.

The role of the mPFC in modulating behavior is widely

encompassing, with only a few functions mentioned above. As such, changes during development, including stress, drugs or social interactions, can affect the organization and function of the mPFC possibly leading to behavioral impairment (Kolb et al., 2012). To this end, our laboratory is interested in investigating the mPFC in the context of developmental disorders. Our specific interest is to understand how the chemistry in this area underlies the behavioral phenotypes of pathologies such as autism spectrum disorder (ASD). Since serotonin plays significant roles in early brain development (Whitaker-Azmitia, 2001) and the mPFC is densely innervated with this messenger's neurons (Robbins, 2005), we find it meritorious to focus our chemical studies on serotonin. Serotonin chemistry in the periphery is impaired during ASD (Hranilovic and Blazevic, 2014) however, there is currently no consensus on how or

Abbreviations: mPFC, medial prefrontal cortex; FSCV, fast-scan cyclic voltammetry; FSCAV, fast-scan controlled adsorption voltammetry; CFM, carbon fiber microelectrode; SNr, substantia nigra, pars reticulata; MFB, medial forebrain bundle; SE, stimulating electrode; WE, working electrode; CV, cyclic voltammogram; IT, current vs. time; Dmn₁, domain 1; Dmn₂, domain 2

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<https://doi.org/10.1016/j.neuint.2018.07.004>

Received 15 May 2018; Received in revised form 22 June 2018; Accepted 13 July 2018

Available online 19 July 2018

0197-0186/ © 2018 Published by Elsevier Ltd.

if the brain's serotonin chemistry, specifically in the mPFC, is altered during ASD. For example, human positron emission studies and animal autoradiographic studies are contradictory in their reports of serotonin transporter (SERT) density and binding capacity, with some reporting decreased SERT binding compared to controls, yet others showing no change in SERT density (Dufour-Rainfray et al., 2010; Nakamura et al., 2010) in ASD cases and models. These studies are then necessarily at odds with other animal-only ASD models that show region-dependent abnormalities in the density of serotonin neurons (Guo and Commons, 2017), reduced SERT binding (Gould et al., 2011) and reduced excitatory transmission (Nakai et al., 2017).

Given the uncertainties outlined above, here we seek to better define serotonin chemistry in the mPFC by applying cutting edge analytical methods, fast-scan cyclic voltammetry (FSCV) and fast-scan controlled adsorption voltammetry (FSCAV), for real-time *in vivo* serotonin analysis in the mPFC. The small size ($\sim 7\ \mu\text{m}$) of the carbon fiber microelectrode (CFM) and excellent selectivity and sensitivity of these techniques allows for sub-second measurements of stimulated serotonin release and reuptake as well as rapid ($< 1\ \text{min}$) ambient level measurements.

Our previous work with FSCV revealed a single phase evoked serotonin response in the substantia nigra, pars reticulata (SNr) upon medial forebrain bundle (MFB) stimulation (Wood et al., 2014). We noted a curious phenomenon in the reuptake profile of serotonin in this region. In some cases, the response would be cleared “fast” ($< 10\ \text{s}$) with a single reuptake curve. In other instances, the response decayed with a “slow” ($> 20\ \text{s}$) profile but in most cases, a combination of ‘fast’ and ‘slow’ clearance profiles resulted in a ‘hybrid’ reuptake. The ‘fast’ and ‘slow’ responses were identified as two distinct clearance mechanisms: Uptake 1, mediated by the serotonin transporters (SERTs), and Uptake 2, mediated by other monoamine transporters (dopamine transporters (DATs), norepinephrine transporters (NETs), and organic cation transporters (OCTs)). These mechanisms had previously been identified and while Uptake 2 was defined as high capacity, low affinity uptake, DATs and NETs can be included since each has multiple types of transporters that band together to simultaneously remove serotonin (Daws, 2009; Shaskan and Snyder, 1970). We mathematically modeled our experimental curves and reported two sets of Michaelis-Menten parameters corresponding to Uptake 1 (high affinity, low efficiency) and Uptake 2 (low affinity, high efficiency).

Here in the mPFC, we found similar single phase evoked responses with a hybrid clearance profile. Additionally, for the first time, we observed two evoked responses to a single MFB stimulation; of particular significance, the two phases had different reuptake profiles. We found a strong correlation between the occurrence of single or dual responses and the position of the CFM within the mPFC layers. Additionally, the magnitude of the second phase in the dual phase response was dependent on the position of the stimulating electrode. These findings allowed us to hypothesize that there are distinct subsets of serotonin axons traversing the MFB, that terminate in discrete domains with specific reuptake profiles. We utilized pharmacology, histology and an elaboration of our prior model in the SNr to test this hypothesis. We subsequently showed that a comprehensive and highly organized cellular network underlies the control of serotonin chemistry in the mPFC. Our findings will, in the future, serve as a base chemical model to study serotonin dysfunction in models of ASD.

2. Materials and Methods

2.1. Animals and surgical procedures

All procedures described herein are in agreement with The Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina (USC). Male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) between 20 and 30 g were used for all stereotaxic surgical

experiments. Mice were housed in the USC animal care facility under a 12-h light/dark cycle with access to food and water *ad libitum*. Experiments were performed during the light phase. Mice were injected with a 25% urethane solution (dissolved in 0.9% saline, Hospira, Lake Forest, IL, USA) intraperitoneally (i.p.) and maintained on a heating pad (Braintree Scientific, Braintree, MA, USA) at 37°C . Fully anesthetized mice were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA) for intracranial surgery. Stereotaxic coordinates were each taken in reference to Bregma (Paxinos, 2012). A Nafion coated carbon fiber microelectrode was lowered into the medial prefrontal cortex (AP: $+1.70$, ML: -0.20 , DV: -2.20 to -2.90) and a stainless-steel stimulating electrode (SE) placed in the medial forebrain bundle (MFB) (AP: -1.58 , ML: -1.00 , DV: -4.80). An Ag/AgCl reference electrode was placed in the contralateral hemisphere (AP: -3.15 , ML: $+1.70$). Placement of the working electrode (WE) was adjusted in the dorsal/ventral plane until the desired serotonin signal was observed.

2.2. Voltammetry

2.2.1. Carbon-fiber microelectrodes (CFMs)

CFMs were fabricated in house using $\sim 7\ \mu\text{m}$ diameter carbon fibers (Goodfellow Corporation, PA, USA). The carbon fibers were aspirated into glass capillaries (ED 0.6 mm, ID 0.4 mm; A-M systems, Inc., Sequim, WA, USA) and pulled via a vertical micropipette puller (Narishige Group, Tokyo, Japan). Under an optical microscope, carbon fibers were cut to $150\ \mu\text{m}$. An electrical connection was forged between the carbon fiber and connection wire using silver paint. CFMs were then electroplated with nafion (Hashemi et al., 2009).

2.2.2. Fast-scan cyclic voltammetry

The serotonin waveform was generated using a PCIe-6341 DAC/ADC (National Instruments, TX, USA) card. The working electrode was scanned at $1000\ \text{V s}^{-1}$ from $0.2\ \text{V}$ to $1.0\ \text{V}$, down to $-0.1\ \text{V}$ then back to $0.2\ \text{V}$ and cycled at $10\ \text{Hz}$ (Jackson et al., 1995). Bi-polar electrical stimulations induced serotonin release ($60\ \text{Hz}$, 120 pulses, $350\ \mu\text{A}$, $4\ \text{ms}$ pulse width), delivered through a linear constant current stimulus isolator. A CHEM-CLAMP potentiostat (Dagan Corporation, MN, USA) measured current. Potentials were measured against a Ag/AgCl reference electrode. A predetermined calibration factor of $49.5 \pm 10.2\ \text{nA } \mu\text{M}^{-1}$ was used to convert current to concentration (Hashemi et al., 2009).

2.2.3. Fast-scan controlled adsorption voltammetry

FSCAV was performed using a CMOS precision analog switch, ADG419 (Analog Devices) to control the application of the computer-generated waveform to the electrode. The logic was controlled programmatically with a series of ramps. The waveform (0.2 – $1.0\ \text{V}$ to -0.1 – $0.2\ \text{V}$, scan rate = $1000\ \text{V s}^{-1}$) was applied at a frequency of $100\ \text{Hz}$ for $2\ \text{s}$, followed by holding at a constant potential ($0.2\ \text{V}$) for $10\ \text{s}$, and finally followed by the recommencement of the waveform for a total file collection time of $30\ \text{s}$ (Abdalla et al., 2017).

2.2.4. Stimulation location experiments

Experiments were carried out to explore the contribution of stimulations along the MFB to the double peak signal amplitude. For these experiments, control files were collected, and the stimulation electrode was then removed from the brain and placed either anterior or posterior to the original placement before being lowered into the brain again. Control files were collected at the secondary SE placement and the procedure was repeated a third time with the SE moved opposite its initial placement (either anterior or posterior). Following collection of control files with the three SE placements, the experiment was terminated.

2.2.5. Analysis and statistics

Custom software (WCCV 3.0, Knowmad Technologies LLC, Tucson, AZ, USA) was used for data collection, processing, and analysis. Data

was background subtracted and filtered at zero-phase using a fourth order Butterworth 5 kHz low pass filter. FSCV yields cyclic voltammograms (CVs) which can be used both qualitatively and quantitatively. CVs are stacked in time to produce a 2-D color plot which allows for extrapolation of current vs. time (IT) plots as well as CVs. IT plots were written as text files and imported to Excel where they were averaged across each time point and converted to concentration using a pre-determined calibration factor (*vide supra*). An average concentration vs. Time trace is generated from this process and the standard error of the mean, taken across each time point, is added to the plot.

FSCAV data was processed and electrodes calibrated as previously described (Abdalla et al., 2017).

2.2.6. Pharmacology

Mice were administered a bolus intraperitoneal (*i.p.*) injection of 10 mg kg⁻¹ escitalopram (ESCIT) (Sigma-Aldrich, St. Louis, MO) during the experiment. Files were collected for up to 2 h following injection.

2.2.7. Exclusion criteria

If the cyclic voltammograms (CVs) collected with FSCV and FSCAV did not match the characteristics of serotonin CVs collected *in vitro*, the mice were excluded from this study. Mice that died before the end of the collection time were excluded. Mice were also excluded if the electrode was found to not be located in the MBF after histology. All other mice were included in this study.

2.3. Histology

Following data collection, a large potential (13 V for 90 s) was passed through the working electrode to lesion the brain. Mouse brains were harvested, following euthanasia, and stored in 4% paraformaldehyde until ready for slice analysis. Brains were transferred to 20% sucrose solution 48 h prior to sectioning. Each brain was sectioned (30 μm) using a cryotome and sections were analyzed for working and stimulating electrode placement. Electrode placements were verified, and mPFC subsection location of the CFM determined based on the Allen Mouse Brain Atlas (Allen, 2004).

2.4. Modeling

Our model for this work is an elaboration of a prior model (Wood et al., 2014), with addition of two domains, which are two distinct types of termini clusters. Domain 1, Dmn₁, is subject to both Uptake 1 and 2 and receives input from Population 1. Domain 2, Dmn₂, subject only to Uptake 1 and receives input from Population 2. Let x be a variable that goes from Dmn₂ ($x = 0$) to Dmn₁, where we expect that L (maximum distance between Dmn₂ to Dmn₁, as estimated from the maximum distance across characteristically similar layers) (Allen Mouse Brain Atlas, 2004) will be of the order of magnitude of 500 μm.

As depicted in Fig. 1, Dmn₂ is the region $0 < x < a$ and Dmn₁ is the region $b < x < L$ where x is a distance variable indicating the position of interest. Let $u(t, x)$ be the extracellular concentration of serotonin at x at time, t . Then $u(t, x)$ satisfies the partial differential equation (1):

$$\frac{\partial u(t, x)}{\partial t} = \text{Input}(t, x) - \text{Uptake}(t, x) + k \cdot \frac{\partial^2 u(t, x)}{\partial x^2} \quad (1)$$

$$u(0, x) = 60 \text{ nM} \quad (2)$$

$$\frac{\partial u(t, 0)}{\partial x} = 0 = \frac{\partial u(t, L)}{\partial x} \quad (3)$$

Where k is the diffusion coefficient of serotonin in the extracellular space. The initial condition (2) states that at time = 0 the system is at steady state and the extracellular concentration of serotonin is equal to 60 nM at all x (estimated from prior models and experimental data

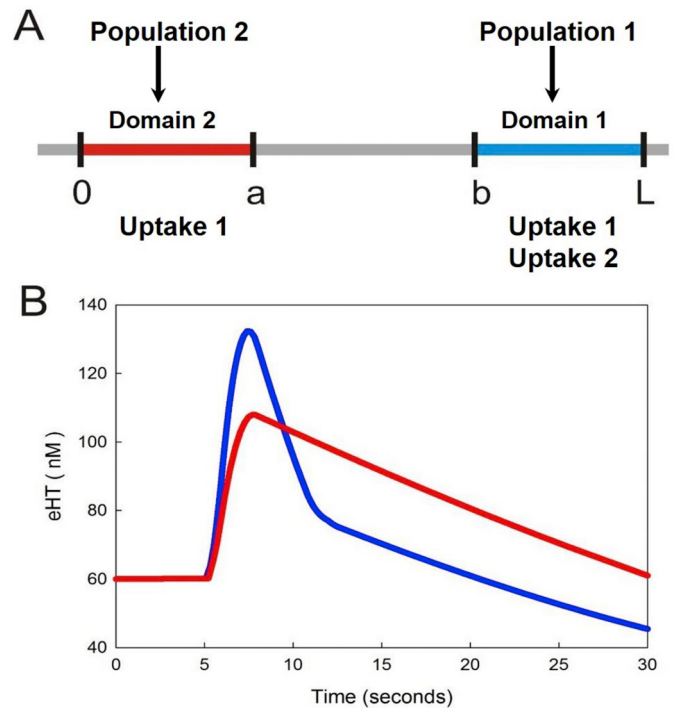


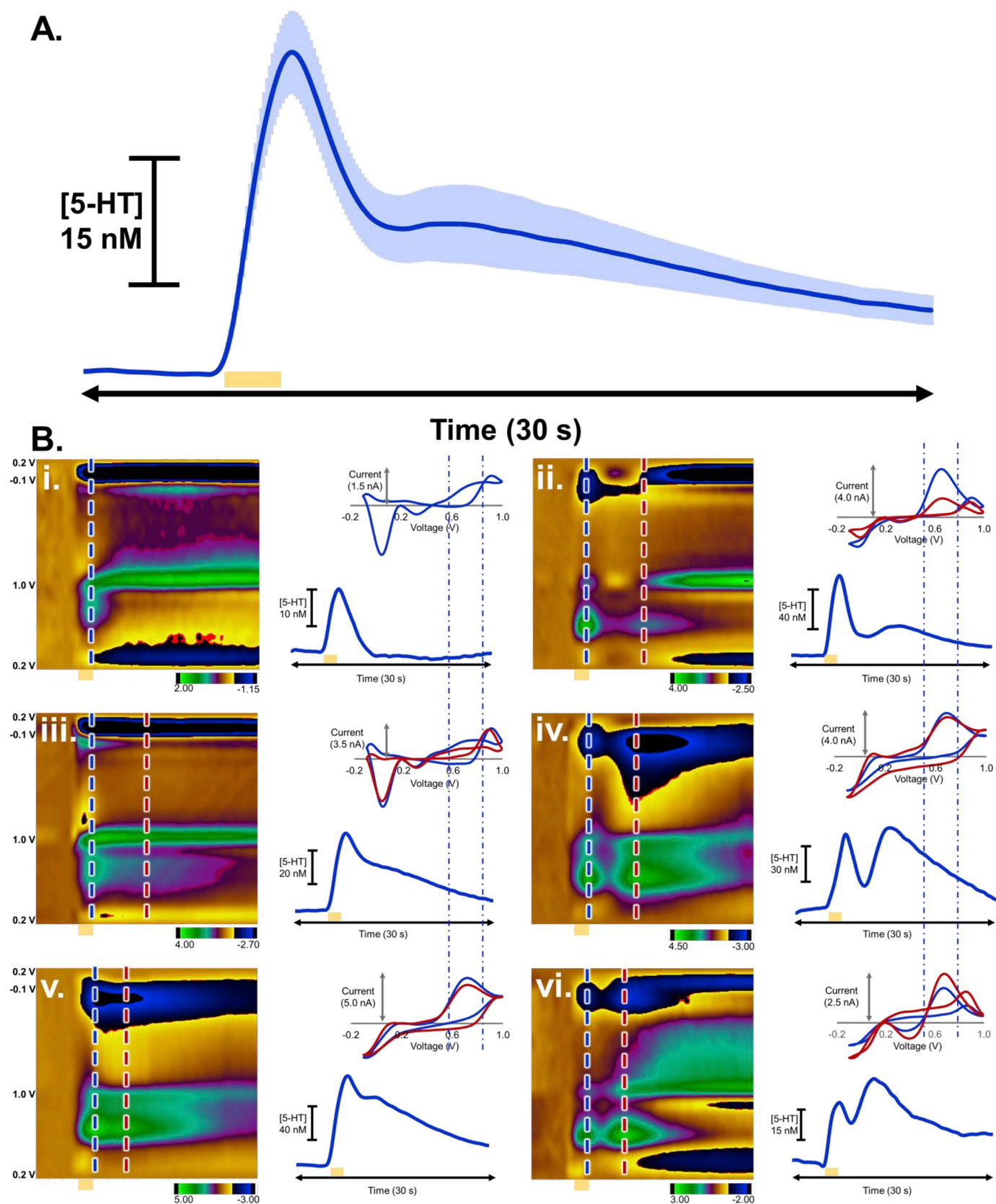
Fig. 1. Representation of the Mathematical Model. Panel A of Fig. 1 shows a geometric sketch of a physiological hypothesis. Population 1 provides input to Dmn₁, located between $x = b$ and $x = L$. Dmn₁ has both Uptake 1 and Uptake 2. Population 2 provides input to Dmn₂, located between $x = 0$ and $x = a$. Dmn₂ has Uptake 1. In the middle region (between $x = a$ and $x = b$), there is no input and no reuptake but extracellular serotonin is free to diffuse. The response (blue) in Panel B shows the time course of extracellular serotonin if only population 1 were stimulated. The response (red) in Panel B shows the time course of extracellular serotonin if only population 2 were stimulated. Experimentally, sole stimulation of population 2 has not been observed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Supplementary Fig. S4) (Abdalla et al., 2017). The boundary condition (3) guarantees that serotonin cannot diffuse out of the interval $[0, L]$.

Input (t, x) is constant at the steady state level until $t = 5$ s, which is the start of MFB stimulation. For a few seconds after $t = 5$, impulses arriving from populations 1 and 2 increase the serotonin concentrations in Dmn₁ and Dmn₂ creating FSCV peaks. After that the peak amplitudes dip below basal level until 30 s because of prolonged autoreceptor effects discussed previously (Wood et al., 2014). The strength of the inputs to Dmn₁ and Dmn₂ depend on the position and strength of the MFB stimulation and are varied in our simulation experiments. There is no input between $x = a$ and $x = b$.

In Dmn₁, Uptake (t, x) is both by Uptake 1 and Uptake 2. Uptake 1 has a Michaelis-Menten profile with $V_{\max 1} = 19.25 \text{ nM s}^{-1}$ and $K_{M1} = 5 \text{ nM}$. Uptake 2 also follows Michaelis-Menten kinetics with parameters $V_{\max 2} = 780 \text{ nM s}^{-1}$ and $K_{M2} = 170 \text{ nM}$. These parameters are almost identical to those we previously established in the SNr (Wood et al., 2014). The constraints of this model include that Dmn₂ is subject to Uptake 1, that Uptake 2 operates only at concentrations above 75 nM, and that there is no uptake between $x = a$ and $x = b$.

The question that we investigate in the simulations in this paper is what will happen in the extracellular space if serotonin can diffuse from type 2 domains (Dmn₂), to the electrode surface, which is enveloped by type 1 domains (Dmn₁). In all of our simulations we assume Dmn₁ are ubiquitous around the electrode. The parameter r , s_1 , and s_2 , are proportional to serotonin release in Dmn₁ and Dmn₂, respectively. Model simulations were performed using MatLab R2017b (MathWorks, Natick, MA, USA) using ODE solver ode23s implemented on an iMAC



(caption on next page)

Fig. 2. Serotonin Responses in the mPFC. The average response of all signals recorded in the mPFC can be seen in (A) with error bars showing SEM. The variety of these signals can be seen in (B) with color plots, concentration vs. time traces, and inset CVs corresponding to the peaks identified from the color plot. The traditional single peak is shown in (i.) along with the variety of double peaks seen in (ii.–vi.). Inset CVs confirm that each peak is serotonin by the oxidation peak at ~ 0.7 V. The correlation between the individual signals and their experiment number (seen in Table 1) is as follows: i.-17, ii.-3, iii.-2, iv.-7, v.-9, vi.-5.

Figure 2A shows the average serotonin response in the mPFC with error bars signifying standard error of the mean for responses averaged between animals. This average response is tallied as a function of two distinct response types. Specifically, in some experiments, we recorded a single evoked serotonin peak with primarily Uptake 2 clearance. However, an equal amount of times we observed a double peak, or dual event, in response to the stimulation. The second peak appeared to be delayed and the amplitude varied with respect to the first. The appearance of these dual responses varied substantially, and representative examples of the five most common types we observed are shown in Fig. 2B. The CVs of both peaks, shown in the inset, clearly identified each release event as serotonin with an oxidation peak around 0.7 V. Capacitive currents were observed on the color plots and CVs at the switching potentials in Fig. 2B, though they did not interfere with serotonin quantification (Samaranayake et al., 2015). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

with operating system OS X version 10.12.6.

3. Results and Discussion

3.1. Two serotonin release events in response to a single stimulation

The MFB was stimulated and serotonin was detected in the mPFC at an implanted CFM. Examples of serotonin release are shown in the color plots in Fig. 2 (*vide supra* for color plot interpretation). This kind of stimulation - measurement method is well established in dopamine voltammetry; for example, dopamine release is induced in the nucleus accumbens upon MFB stimulation (Hashemi et al., 2012). It is generally well accepted in the voltammetry community that MFB stimulated dopamine release, which is abolished *via* lidocaine-induced cell body inactivation is antidromic (Somers et al., 2009). We believe that this principle applies to MFB stimulated serotonin release in the mPFC.

To the best of our knowledge, this is the first time that biphasic responses have been observed with serotonin. Dopamine has been previously observed, with FSCV, to exhibit a variety of profiles depending on measurement location, though the reasoning for these responses cannot wholly be applied to this instance for serotonin (Walters et al., 2015).

While several hypotheses were considered for this biphasic phenomenon, including mobilization of different vesicular pools and second messenger effects, we believe that the data are best explained via a combination of two theories. First, parallel projection systems have previously been postulated to underpin dopamine circuitry from the ventral tegmental area (Ikemoto, 2007). Likewise, parallel serotonin pathways from the dorsal and median raphe nuclei project to the mPFC (Vertes, 1991; Vertes et al., 1999). Second, biochemically distinct compartments form the basis of the striosome-matrix hypothesis (Brimblecombe and Cragg, 2015; Gerfen et al., 1987; Graybiel et al., 1981). Thus, here we hypothesize two different, parallel populations of serotonin axons in the MFB that terminate in different biochemical compartments (not to be confused with mPFC layers), which here we term domains, Dmn₁ and Dmn₂. Dmn₁ receives input from population 1 and clearance occurs *via* both Uptake 1 and Uptake 2. Dmn₂ receives input from population 2 and the serotonin released here is cleared *via* Uptake 1. An illustration of this hypothesis, with two scenarios illustrating the CFM location either in layers 1–3 or in layers 5–6, can be found in Fig. 3. The prospect of Uptake 2 clearing serotonin is substantiated as DATs, NETs, and OCTs have been found in the mPFC. While rodent studies have shown a higher expression of SERTs compared to DATs and NETs in the mPFC (Tellez et al., 2012), their high efficiency allows them to suitably clear serotonin. OCTs have been found with some abundance in the mPFC (Gasser et al., 2009) but information regarding their expression relative to DATs and NETs is less available.

Our experimental data (*vide infra*) suggests that Dmn₂ occurs sparsely across layers and that Dmn₁ is ubiquitous. The sparse innervation of Dmn₂ is such that we believe that our electrode is never close enough to detect peaks from Dmn₂ first, but the ubiquitous nature of Dmn₁ makes our electrode constantly consistently surrounded by this domain. Our hypothesis is that the second peak is caused by the diffusion of

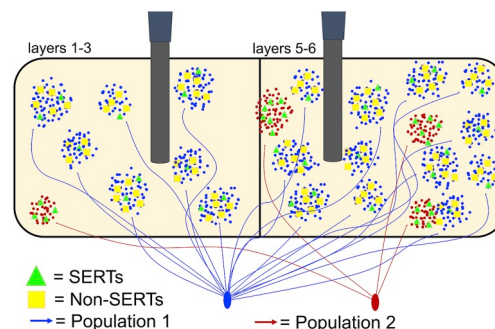


Fig. 3. Theory Schematic. Population 1 (blue) has input to all layers and terminates in Dmn₁ with Uptake 1 (SERTs, green triangles) and Uptake 2 (non-SERTs, yellow squares) transporters. Population 2 (red) has input primarily to layers 5 and 6 and terminates in Dmn₂ where Uptake 1 dominates. The time delay of the second peak is due to diffusion, since Dmn₂ is more sparse, thus farther from the CFM. This phenomenon also explains why the second peak is only observed in layers 1–3 after administering ESCIT (see Fig. 5); where amplitude of serotonin released in Dmn₂ is now high enough to be measured. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

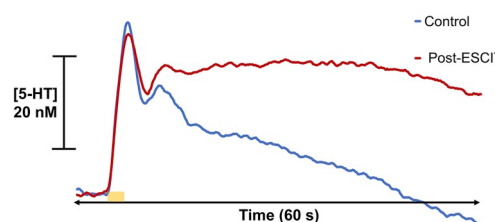


Fig. 4. A Double Peak pharmacological experiment. A representative response (experiment 18) is shown before (blue) and after (red) *i.p.* Administration of 10 mg kg⁻¹ ESCIT. Both curves show a biphasic response. Our hypothesis of diffusion from Dmn₂ to Dmn₁ explains the differences between the curves. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

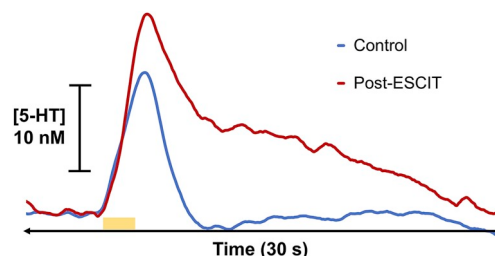


Fig. 5. A Single Peak Pharmacological Experiment. The [5-HT] vs. time traces are shown for a representative (experiment 15) single peak serotonin response before (blue) and 60 min after (red) 10 mg kg⁻¹ ESCIT administration. Histology confirmed that the CFM was in layers 1–3. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

serotonin from Dmn₂, via activation of population 2, to the electrode surface. We now explore this notion in more detail.

Fig. 4 shows data from a representative experiment where a dual response is observed pre (blue trace) and post (red trace) 10 mg kg⁻¹ ESCIT administration. Pre-drug, the extracellular serotonin rises rapidly after the stimulation followed by a rapid, Uptake 2 mediated, decline. At about t = 11 s the curve begins to rise again because of diffusion of serotonin from Dmn₂ to the CFM. In this example the initial reuptake profile of the second peak is primarily mediated by Uptake 2 as serotonin from Dmn₂ has diffused to the electrode surface which is surrounded by Dmn₁ and the high concentrations of serotonin have activated Dmn₁ Uptake 2 transporters. Once the concentration falls below a certain threshold, Uptake 2 ceases and Uptake 1 dominates. Post ESCIT, the first peak is not largely affected because ESCIT does not significantly target Uptake 2 transporters (Brennum et al., 2002). The second peak, conversely, increases in amplitude and the decay is significantly slowed, because clearance here is mostly mediated by Uptake 1 (SERTs). This response to ESCIT was a consistent trend seen across the dual response signals obtained. An additional representative experiment with these conditions can be found in the Supplementary Material (Fig. S1).

These results confirm that the two individual peaks are selectively associated with different uptake mechanisms. This uptake specificity exists regardless of whether a second peak is observed or not. Above we hypothesized that the two peaks result from activation of separate populations and explore this in more detail in the following sections.

3.2. Single or dual evoked response are dependent on sub-region layers

In defining the origin of the two evoked serotonin peaks, we first explored the correlation of the position of the CFM in the mPFC tapestry to the prevalence of single vs. double peaks. Following data collection, a large potential was applied to the working electrode, which we previously found successfully created an identifying lesion in the brain tissue (otherwise undetectable due to the miniature dimensions of the CFM). The lesions were categorized on the sub region (prelimbic, infralimbic, and anterior cingulate cortex) and layer (1–6) of the mPFC. We found no trend between sub regions, but single vs. double peaks were distinctly dependent on layer. Table 1 correlates the signal type

Table 1

Correlating Peak Response to Measurement Location. The table correlates the number of peaks (one or two) with the sub-region (anterior cingulate, prelimbic, or infralimbic cortex) and layer (1–6) of the mPFC in which the CFM lesion was found for every experiment included in this paper. Single peaks were consistently only found in layers 1–3, while double peaks were only found in layers 5–6.

Experiment Number	Number of Peaks	WE Sub-region	WE Layer
1	Two	Anterior Cingulate	5
2	Two	Prelimbic	5
3	Two	Anterior Cingulate	5
4	Two	Anterior Cingulate	5
5	Two	Anterior Cingulate	6a
6	One	Anterior Cingulate	2/3
7	Two	Prelimbic	5
8	One	Infralimbic	2/3
9	Two	Anterior Cingulate	5
10	One	Anterior Cingulate	2/3
11	One	Infralimbic	1
12	One	Infralimbic	1
13	One	Infralimbic	1
14	Two	Anterior Cingulate	5
15	One	Prelimbic	2/3
16	One	Anterior Cingulate	1
17	One	Infralimbic	1
18	Two	Infralimbic	5
19	One	Anterior Cingulate	2/3
20	Two	Prelimbic	5

(single vs. double) with the sub-region and layer in which the CFM made measurements. Visual representation of the histology can be found in the Supplementary Material (Fig. S2).

Double peaks were consistently observed in layers 5–6, while single peaks were seen only in layers 1–3. This trend was consistent across all subregions of the mPFC. While layers are traditionally defined by the synaptic density (Andrade, 2011), the data shown here supports the theory that the layers may also define the topographical limits of functionally different serotonin neurons. In support of this, morphological differences between cortex varicosities have been identified (Kosofsky and Molliver, 1987). These different varicosities can be directly associated with specific projections from the dorsal and medial raphe which have been shown to terminate in a layer dependent manner (Kosofsky and Molliver, 1987; Vertes, 1991; Vertes et al., 1999). In addition, there is genetic (Kiyasova et al., 2011) and electrophysiological (Hajos et al., 1998) evidence for functionally different serotonin neurons in the frontal cortex.

An interesting phenomenon was observed following administration of ESCIT in experiments where only single peaks were observed; a second peak could emerge after ESCIT. Fig. 5 shows a representative experiment where single evoked events were measured before (control, blue) and 40–60 min after (red) ESCIT. Histology confirmed that the CFM was located in layer 1–3, as above for single peaks. An additional representative experiment with the same conditions can be found in the Supplementary Material (Fig. S3).

The experiments listed in Table 1 revealed that the occurrence of single or dual evoked events are dependent on the layer in which the CFM is located. Fig. 5 experiments indicated layer 1–3 have the potential for the second peak, but that prior to ESCIT the amplitude is too low to be detected. The capacity for a second peak provides support for the presence of Dmn₂ in layers 1–3, though much more sparse than in layers 5–6, resulting in an amplitude too low to be detected without pharmacological intervention.

We observed another phenomenon related to the dual response. In different animals there was a large variance in the ratio of the amplitude of the second to first serotonin peak even when the CFM was in the same layer and sub region, pointing towards a important role for the stimulation location. We next investigated this notion.

3.3. Second peak amplitude is heavily dependent on MFB stimulation

To further define the origin of the two evoked serotonin peaks, we test the hypothesis that the two evoked serotonin events occur via stimulation of separate axonal populations, traversing the MFB. We found that stimulating different locations along the MFB, in the AP axes, changed evoked serotonin amplitude, with the most significant effect on the second peak. We postulate that these stimulation electrode placements have less effect on the first peak because axonal population 1 must be more consistently located along the MFB tract that we probed.

3.4. Modeling two discrete axonal populations that terminate onto specific transporter domains

Fig. 2B shows five different experimental response curves measured in layers 5–6, and one response measured in layers 1–3 of the mPFC. Our fundamental hypothesis is that there are two types of domains in layers 5 and 6: Dmn₁ is innervated by population 1 of axons from the MFB and once released into this domain, serotonin is reuptaken by Uptake 1 and 2 transporters. Dmn₂ is innervated by population 2 axons and once serotonin is released into this domain, it is uptaken by Uptake 1 transporters. We experimentally demonstrated that Dmn₁ is ubiquitous and Dmn₂ occurs more sparsely. Therefore, we assumed that the CFM always measures serotonin released from Dmn₁. The potential for the CFM to measure serotonin released from Dmn₂ depends on several factors including: input to population 2, the diffusion length L, and

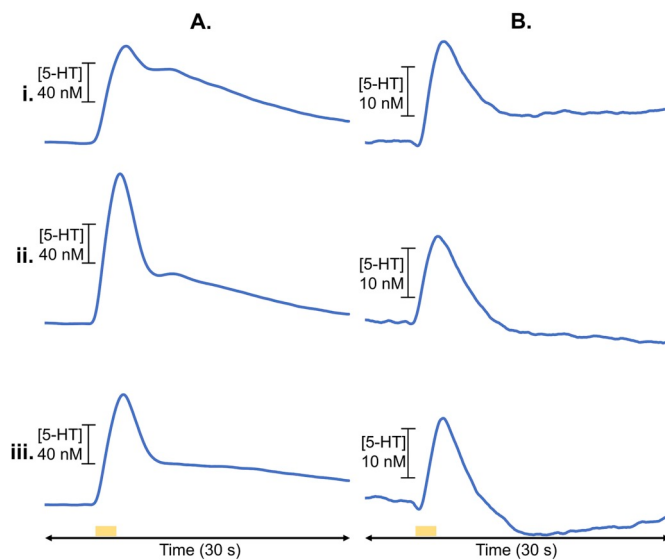


Fig. 6. Investigating the Effects of Stimulation Location. The left panel (A) shows a serotonin double peak response in a single animal (experiment 9). The right side (B) shows a serotonin single peak response in a single animal (experiment 11). In each experiment, control files were collected with three different SE placements (i.-iii.) while the WE remained in the same place. The SE was removed from the brain and reinserted twice, anterior (i.) and posterior (iii.) to the original (ii.) placement.

Figure 6A depicts three double peak signals obtained in one animal. Each signal was collected by stimulating a different location along the MFB moving anterior (i) to posterior (iii). The second peak amplitude increases with the anterior placement of the SE and decreases posterior. In the third signal (iii), and most posterior SE placement, the second peak is almost completely eliminated. In Fig. 6B the experiment was repeated when a single peak was observed, and movement of the SE did not cause a double peak to appear. This allows us to conclude that while the stimulation along the MFB controls the amplitude of the second peak, the presence of a single or double peak remains wholly dependent on the CFM placement within the mPFC layers. We next study the discrete reuptake mechanisms tied to each domain.

coefficient k . As the serotonin released from Dmn_2 diffuses through the extracellular tissue it is reuptaken by the transporters of surrounding domains, thus its reuptake profile is heavily dependent on the extracellular serotonin concentrations. In the **Materials and Methods** Section 2.4, we formulated a mathematical model based on these hypotheses. Our mathematical model fails if serotonin from Dmn_2 was either cleared exclusively by Uptake 2 or initially cleared by Uptake 2 and then subsequently cleared by Uptake 1.

Our model represents a highly complex physiological system and the intent is not to exactly match the experimental curves in Fig. 2B. Rather, the purpose is to show, using the model, that by varying s_1 and s_2 , the strengths of the stimulation from populations 1 and 2, respectively, the diffusion length L and coefficient k , and the basal extracellular serotonin, that we can obtain the variety of behaviors seen in the experimental curves in Fig. 2. We next discuss the response and model curves (i)-(vi) individually. Of note: although the aspect ratios of the curves are the same, the scale of the y-axis varies in both the experimental curves (Fig. 2B) and the model curves (Fig. 7).

Response (i). The single peak simulated here arose from stimulation of population 1 ($s_1 = 0.5$), uptaken rapidly, indicating mainly Uptake 2 mediated transport (Wood et al., 2014). To fit this peak the following parameters were implemented: diffusion coefficient $k = 0$, indicating no diffusion took place from Dmn_2 to Dmn_1 where the CFM is located. The basal level of serotonin in the extracellular space was assumed to be in the higher range, making Uptake 2 operational. Thus Uptake 2 quickly brought the released serotonin back down to baseline. The curve then descended below baseline as a result of the autoreceptor

effect previously discussed (Wood et al., 2014).

Response (ii). For this simulation, population 2 was stimulated in addition to population 1 resulting in diffusion of serotonin from Dmn_2 to the electrode surface, resulting in two peaks. The first peak descended quickly because the extracellular serotonin is within the range which Uptake 2 operated. The second peak was reuptaken more slowly by the Uptake 1 transporters as the extracellular serotonin descended below the range of Uptake 2, in addition to the fact that serotonin continued diffusing from Dmn_2 .

Response (iii). This resembled a typical hybrid response previously observed (Wood et al., 2014), with the exception that a second peak shortly followed the first. To simulate this response, the stimulation of population 1 was $s_1 = 0.6$, and that of population 2 was $s_2 = 1$. The basal level of serotonin was in the middle range. The model then produced a hybrid response with a slight second peak that occurred close to the first peak as the diffusion length between the two domains was reduced by 33%.

Response (iv). The simulation shown here had input to population 1, $s_1 = 0.8$, and input to population 2, $s_2 = 17$. This resulted in a lower amplitude of the first peak and higher amplitude of the second peak. The reuptake of the second peak was faster in this simulation as a result of the extracellular serotonin remaining in the range necessary for Uptake 2 to function up to $t = 30$ s.

Response (v). This response was simulated in the model by setting the input of population 1, $s_1 = 0.8$, and population 2, $s_2 = 2$, and reducing the diffusion length between Dmn_1 and Dmn_2 . The basal level of serotonin was in the lower range so serotonin was reuptaken by Uptake 1 transporters, because Uptake 2 transporters were inactive.

Response (vi). In the experimental curve in Fig. 2Bvi, which this simulation resembles, the first peak was lower in amplitude than the second, while the second peak occurred earlier in time than in the other curves shown. This effect was produced in the model simulation with input of the population 1, $s_1 = 0.6$, and shortening the diffusion length from Dmn_2 to Dmn_1 . The resulting curve showed a pulse of extracellular serotonin from Dmn_2 arriving earlier to the electrode, in Dmn_1 .

These simulations, generated with our new model, described the features of the experimental serotonin responses observed in the mPFC. Additional simulations (not shown) with varying parameters can give a multitude of other single and double-peak shapes of response curves. Thus, this mathematical model accurately supports the hypothesis that we constructed based upon our experimental data.

4. Conclusion

The medial prefrontal cortex (mPFC) plays a critical role in a variety of functions and behaviors. In working towards characterizing the functionality of the mPFC, we directed our efforts towards delineating serotonin transmission in this area. The application of FSCV and FSCAV allowed us the capacity to, for the first time, produce real-time *in vivo* serotonin measurements in the mPFC. We had previously modeled evoked serotonin release in the substantia nigra, pars reticulata, and identified two clearance mechanisms: Uptake 1 and Uptake 2, each mediated by different transporters (Wood et al., 2014). Our current work in the mPFC, presented here, revealed two distinct evoked serotonin events, resulting from a single stimulation of the MFB, which were each bound to a discrete reuptake profile. We hypothesized that distinct populations of serotonin axons traverse the MFB and terminate in discrete reuptake domains. This hypothesis was explored and substantiated via pharmacology, histology, and mathematical modeling. In this work, we presented evidence for the complex organization and regulation of serotonin transmission in the mPFC. The model developed from this work lays the groundwork for our future studies of the function of the mPFC and its relation to behavioral outcomes.

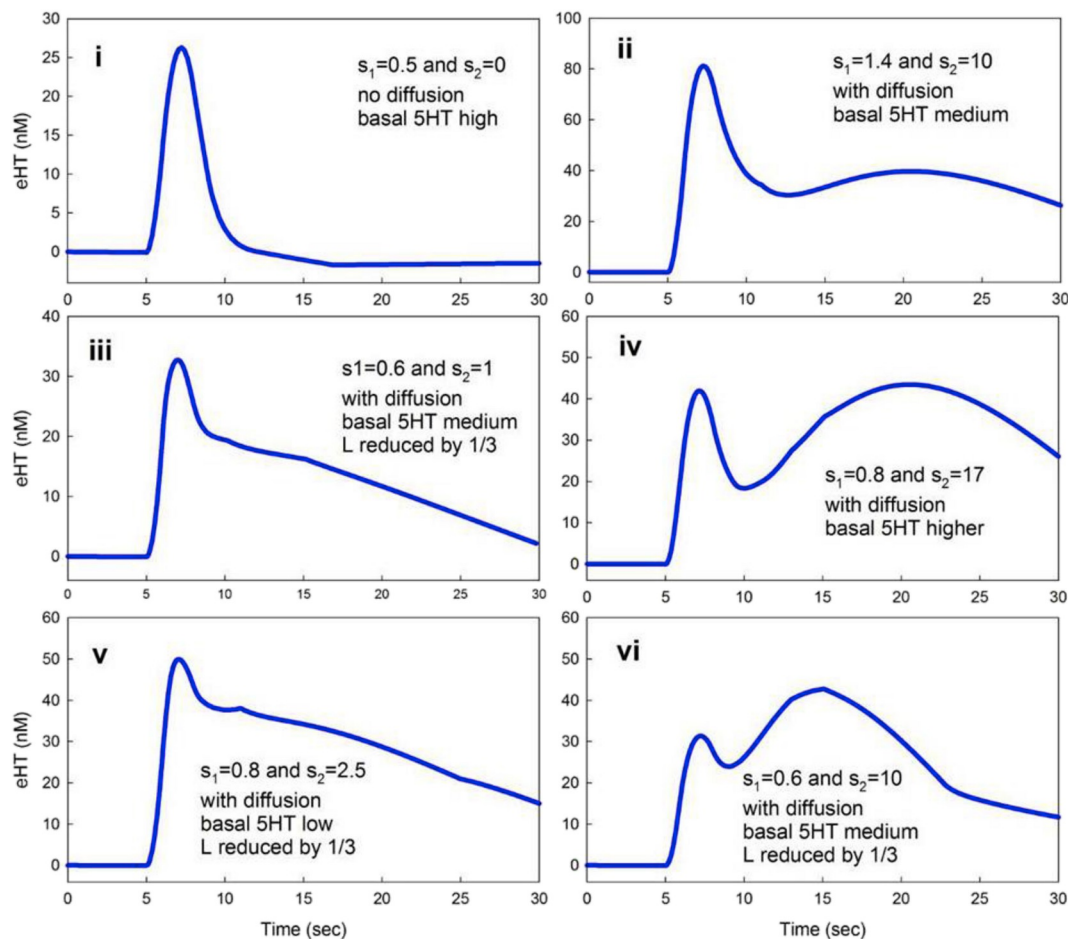


Fig. 7. Model simulations. Panels i–vi show the extracellular serotonin response with different choices of parameters. S_1 and S_2 represent the strength of the neural inputs to Domains 1 and 2, respectively. In Panel (i) there is no diffusion, so the response only represents serotonin released from Dmn_1 . In Panels (ii), (iii), (iv), (v), and (vi) the diffusion coefficient is the same, $k = 0.3 \text{ (nM)}^2 \text{ s}^{-1}$. In each panel the approximate basal level of extracellular serotonin is indicated. We have shown, using FSCAV, that basal serotonin levels in the mPFC vary over a range of 50–75 nM (see Supplementary Materials Fig. S4). In Panels (iii), (v), and (vi) the diffusion length (L) from Dmn_2 to Dmn_1 is reduced by 33%. These simulations can be compared with experimental curves in Fig. 2B.

Funding

This work was supported by the National Institutes of Health R01MH106563 (PH, JB, MCR and HFN), and the Eli Lilly Young Investigator Award (P.H.).

Acknowledgements

The authors would like to thank Navid Tavakoli for experimental assistance and Melinda Hersey, Shane Berger, and Jordan Holmes for their input to constructive discussions and assistance with proofreading this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.neuint.2018.07.004>.

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