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Brain Invaders: Exploring the Impact of Platelet Transmigration on Serotonin Signaling in the Medial Prefrontal Cortex

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ABSTRACT

Brain Invaders: Exploring the Impact of Platelet Transmigration on Serotonin Signaling in the Medial Prefrontal Cortex

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Recent evidence in immunology has indicated that a peripheral player may be contributing to central serotonergic signaling. Researchers have shown that transmigration of blood platelets across the blood brain barrier (BBB) occurs during times of inflammation in the CNS, but none have suggested whether or not this process impacts central serotonin signaling. This paper addresses current research determining the transmigration of platelets into the CNS under natural conditions and addresses future work to solidify any contribution by platelets to CNS 5-HT signaling. Using immunohistochemistry and confocal imaging we mapped the serotonergic varicosities of the mouse brain and used them in comparison to a swellshark (lacking a peripheral serotonin system) to determine any significant differences. It was observed that the 'loose-varicosities' seen in the mouse did not appear to be present in the sharks, indicating that these may actually be transmigrated platelets. In order to determine whether or not platelets that cross the BBB contribute significantly to 5-HT signaling we must first determine an accurate and efficient sensor to extracellular changes of 5-HT. In the presence of Fluoxetine (a serotonin reuptake inhibitor), RTq-PCR was used on a variety of

receptors to determine sensitivity. Findings were inconclusive for all but one receptor (ITGB3), showing a significant increase in mRNA transcripts. Our results require further verification of platelets from the microvasculature in the CNS using more definitive labeling techniques, elucidation of additional markers as efficient serotonin sensors, and assessing changes in receptor expression as a function of pharmacological platelet depletion.

INTRODUCTION

The function of the medial prefrontal cortex (mPFC) strongly depends on serotonin signaling. The biogenic amine serotonin, also known as 5-hydroxytryptamine (5-HT), is a major neurotransmitter in the central nervous system (CNS). The activity of projection neurons and interneurons within the mPFC are controlled by a number of serotonin receptors that directly affect their firing rates. Serotonergic activity within the mPFC has been associated with multiple important functions including working memory, attentional processes, and decision-making. Currently, studies concerning altered 5-HT levels and their role in neurological diseases focus on 5-HT produced strictly in the CNS. However, the brain only synthesizes and contains around 5% of the body's 5-HT. The remaining 95% is produced by the small intestine, and it is currently believed that this peripheral 5-HT never finds its way into the brain. This is due to the protective function of the blood brain barrier (BBB), which is currently known to block 5-HT entry. Thus, the central and peripheral 5-HT systems are considered to be functionally separate from one another. This paper will discuss the possibility that the predominant 5-HT source in the periphery of the body affects 5-HT signaling within the brain via blood platelet transmigration.

5-HT signaling in the mPFC

Central 5-HT axons terminate onto widely distributed locations throughout the brain. One region targeted by raphe 5-HT terminals is the mPFC, which contains a high density of 5-HT receptors and these are modulated in a region-specific manner. Research using rodent models to describe the function of the human mPFC has brought to into question the validity of comparing the cortex of rodent brain and primate brains. The homology between the primate and rodent mPFC is a highly contentious subject, but strong evidence exists that the

rodent mPFC is functionally homologous to the primate agranular mPFC (Uylings, Groenewegen, & Kolb, 2003). The mPFC in the rat contains four main subdivisions: the medial agranular (AGm), the anterior cingulate (AC), the prelimbic (PL), and the infralimbic (IL) cortices (Vertes, 2004). These subdivisions are represented by distinct patterns of connectivity, which feed into the various cognitive and emotional roles associated with each cortical node. Even within a single brain region, differential IL and PL projections can be observed. For example, it has been suggested that the PL and IL play opposing roles in fear behavior regulation. The PL is thought to promote fear expression by increasing amygdala output, while the IL has an inhibitory effect on amygdala output (T. Chan et al., 2011). Findings in adult rats agree with this model: microstimulation of the PL increases conditioned freezing and impairs extinction, while microstimulation of the IL decreases freezing and facilitates extinction under standard footshock conditions (Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006).

The mPFC is rich with both dense intra-connectivity between different nodes within the mPFC itself and outward projections to termination sites distributed throughout the brain. Altered 5-HT function within these networks is thought to play a role in the pathophysiology of depression (Sullivan et al., 2009), schizophrenia (Muguruza et al., 2013), and drug addiction (Liu, Bubar, Lanfranco, Hillman, & Cunningham, 2007). The main area of 5-HT synthesis in the brain is the dorsal raphe nucleus (DRN), which is controlled by several forebrain regions, including the mPFC. The mPFC and DRN form an active circuit within this network and the communication between the two regions has been studied heavily for its role in depression. This cortical projection is mainly glutamatergic and exerts a top-down control over 5-HT neurons in the DRN. With recent advances in optogenetics researchers can

use light to precisely control specific neurons in this pathway. Melissa Warden and colleagues were able to demonstrate that optogenetic stimulation of the mPFC-DRN pathway induces immediate anti-depressant effects as measured by increased kick frequency during the forced swim test (Warden et al., 2012). A later study reported that not only are more of these glutamatergic cortical projections synapsing onto GABA cells in the DRN to regulate 5-HT output, but that optogenetic stimulation of the same pathway increases social avoidance in chronically defeated rats (Challis et al., 2013). The results of these two studies are at odds with one another, however, this contradiction could be a result of the inherent differences between the models employed. The forced swim test and social interaction task could be engaging different defense systems (flight vs. behavioral inhibition), each of which may be dissimilarly regulated by 5-HT. Nevertheless, the studies both implicate the circuit in the regulation of behavioral responses to aversive challenges.

More generally, cortical 5-HT is a key player in decision-making, a daily activity that results from the confluence of cognitive and affective functions (Homberg, 2012). Decisions routinely initiated by high impulsivity can be a hallmark feature of multiple neuropsychiatric conditions such as substance abuse disorders (Verdejo-García, Lawrence, & Clark, 2008). Work done using stable high impulsive and low impulsive phenotypes from an outbred rodent population, found a positive correlation between impulsive action (measured with the 1-choice serial reaction time task) and 5-HT_{2a}R binding density in the mPFC by quantifying [^{3}H]-ketanserin radioligand binding (Fink et al., 2015). With individual variation in inherent impulsivity playing a role in pathological behaviors, it is not surprising that parallel dysregulation of 5-HT_{2a}R binding was discovered in postmortem PFC of schizophrenic subjects untreated with anti-psychotics (Muguruza et al., 2013). Similar dysregulation has

been observed in the PFC of suicide victims, establishing a link between increased 5-HT_{2a} receptor expression and suicidality (Oquendo et al., 2006; Pandey et al., 2002). Central serotonergic signaling has an enormous range of behavioral influence within the mPFC, but the role of 5-HT also extends to a number of biological processes within the periphery.

Peripheral 5-HT system

Outside the CNS, 5-HT is a vital gastrointestinal (GI) signaling molecule within the enteric nervous system (ENS). Enterochromaffin cells (EC) within the gut epithelium synthesize 90% of the total amount of 5-HT in the body, while the remainder is produced by ENS neurons (Janušonis, 2014b). 5-HT is synthesized in two steps, with tryptophan hydroxylase (Tph) as the rate-limiting enzyme (Walther, 2003). The two forms of Tph, Tph1 and Tph2, are found, respectively, in EC cells and neurons (Gershon & Tack, 2007). 5-HT plays two distinct roles within the gut. First, gut 5-HT can act as a neurotransmitter in the ENS, exerting its action within the myenteric and submucosal plexuses. ENS neurons use 5-HT to carry out several vital functions: they modulate GI secretion, intestinal motility, and pain sensitivity (McLean, Borman, & Lee, 2007). Ultimately, 5-HT signaling must be terminated via uptake by other GI cells. Extracellular 5-HT is taken up by neurons and enterocytes (non-neuronal absorptive cells present in the gastrointestinal mucosa) that express the serotonin reuptake transporter, SERT (Gershon, 2004). SERT is a molecular pump embedded in the plasma membrane of particular cells and modulates the strength and duration of 5-HT signaling. It is often studied in reference to the CNS because it is a common target of the psychoactive drugs used to treat depression (Whyte, Jessen, Varney, & Carneiro, 2014). However, SERT is expressed by several peripheral tissues, including: lung endothelial cells, gastrointestinal epithelium, systemic arteries and veins, the adrenal gland,

lymphocytes, and in the placenta and blood platelets (Yubero-Lahoz, Robledo, Farré, & de laTorre, 2013).

The second role of gut 5-HT is initiated when gut 5-HT enters the systemic blood circulation, where most of this free 5-HT is cleared by organs such as the liver and lungs (Aster, 1966). Some of the free 5-HT is taken up by circulating blood platelets (small non-nucleated cell fragments) expressing SERT. Blood platelets are an indispensable component of the blood, where they play important roles in blood clotting and regulation of vascular tone (Esmon, 1993). Circulating platelets carry 5-HT throughout all organs including the brain, which is interlaced with a dense network of capillaries. Though platelets carrying 5-HT are extremely abundant within the brain vasculature, it is widely accepted that platelets do not penetrate the BBB and thus do not participate in central 5-HT signaling. It is noteworthy that mammals are the only animals that possess blood platelets. Non-mammals with systemically circulating 5-HT carry peripheral 5-HT within nucleated blood cells called thrombocytes (Greenberg et al., 1999). Some vertebrates do not contain any 5-HT molecules in their blood. For example, the blood of fishes is completely devoid of peripheral 5-HT but the fish central 5-HT system is homologous to that of vertebrates (Levin, 2002). This puts fishes in the position to provide an ideal model system to study a natural CNS serotonergic environment and its vasculature that is unaffected by peripheral 5-HT. Within the mammalian systemic blood circulation, peripherally derived 5-HT is abundantly present and could only affect the central 5-HT system if it was able to traverse the vascular endothelial cells of the BBB. The 'neurovascular unit' is more recently considered synonymous with the BBB, to indicate that the endothelium dynamically expresses its barrier properties by collecting input from other cell types, such as neurons and astrocytes within the brain

parenchyma (Mäe, Armulik, & Betsholtz, 2011). The BBB is indeed a dynamic entity, whose permeability can be manipulated by pharmacological agents as well as the endogenous molecules elicited by the stress response (Sharma, 2004). Yet, it is currently believed that this permeability is never extended to 5-HT molecules.

Potential transmigration

Recent studies have challenged the perceived impermeability of the BBB to 5-HT, suggesting that transmigration is possible for 5-HT molecules. One lab has found that elevated brain 5-HT levels can cause a significant rise in blood 5-HT levels (Nakatani et al., 2008). While the notion of peripheral 5-HT infiltrates is rather controversial, the field of immunology has established that some cells can certainly escape capillaries and reach the neurons normally protected by the BBB. Recent studies have shown that specialized large blood cells, monocytes, are able to enter into the brain parenchyma under neuroinflammatory conditions such as those induced by social stress. Social stress models in rodents, such as repeated social defeat (RSD), mimic the immunological and behavioral conditions linked to psychosocial stress in humans. RSD activates stress-responsive brain regions associated with fear and anxiety that seem to cause the behavioral adaptations associated with experiencing high levels of social threat (Reader et al., 2015). One recent study demonstrated that RSD in mice causes an increase in mRNA expression of vascular adhesion molecules and chemokines associated with monocyte recruitment, and that these increases are localized to the vasculature of the distinct brain regions implicated in fear and anxiety responses (Sawicki et al., 2014).

Neuroinflammation is also associated with HIV-1 infected individuals, presenting another opportunity to study transmigration occurring when the BBB is compromised. One

research group found that blood platelets have been observed "piggy-backing" on transmigrating monocytes and that these transient platelet-monocyte complexes (PMCs) were increased in the whole blood of HIV-1 patients, quantified with flow cytometry. Furthermore, *in-vitro* culture assays revealed that PMCs display increased adherence to endothelial cells and enhanced transendothelial migration (Singh et al., 2014). These pieces of information do not prove that platelets are leaving circulation, yet they do suggest that platelets are able to cross the BBB under certain conditions. A more conservative interpretation would be that platelets are increasing the permeability of the BBB to monocytes through their action as inflammatory mediators, given that platelets are able to release 5-HT into the extracellular space through a process called degranulation, which can act on 5-HT_2 receptors in the CNS microvasculature to increase BBB permeability (Abbott, 2000). However, given that blood platelets are subcellular in size, it is reasonable to question whether they too can transmigrate.

Recent research in immunology has revealed that platelets recognize specific glycolipid structure called gangliosides, situated in lipid rafts on the surface of astroglial and neuronal cells. Activated receptors on platelet surfaces, such as CD62P, enable platelets to recognize these distinct ganglioside formations and release proinflammatory factors such as 5-HT into extracellular space (Sotnikov et al., 2013). These results suggested that platelets alone were able to enter mouse brain parenchyma and interact with brain cells (neurons and astrocytes), yet this process has not been established definitively and further investigation is needed. Based on this emerging evidence, platelets could be transmigrating into the brain parenchyma and communicating with neuronal and astroglial cells. Moreover, transmigrating blood platelets may be mistaken for 5-HT varicosities. Serotonergic

varicosities and blood platelets share both obvious and subtle similarities. Both contain 5-HT, are approximately the same size (Benzekhroufa, Liu, Tang, Teschemacher, & Kasparov, 2009), lack nuclei, and are located remote distances away from the cell bodies that have produced them (Fig. 1). Both platelets and varicosities express SERT, 5-HT receptors, and integrin β_3 (ITGB3) (Whyte et al., 2014). In immunology, CD61 is commonly used as a selective platelet marker (Singh et al., 2014; Sotnikov et al., 2013) and has been used to track the migration of platelets. Emerging evidence in immunology supports the concept of platelet transmigration, however, these studies do not consider the potential effects of platelet transmigration on brain 5-HT signaling. This paper will explore the possibility that peripheral 5-HT from blood platelets can affect central 5-HT signaling within the mPFC via transmigration.

METHODS

Aim 1: Characterize mPFC serotonergic varicosities and platelet profiles

Animals

Adult C57BL/6J mice and timed-pregnant C57BL/6J mice were purchased from The Jackson Laboratory and housed individually on a 12:12 light-dark cycle with free access to water and food. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

The heads of adult swellsharks (*Cephaloscyllium ventriosum*) were obtained from the Parasitology laboratory taught at the UCSB Department of Ecology, Evolution and Marine Biology (EEMB 111) and immediately stored on ice.

Immunohistochemistry

Collagen IV immunohistochemistry with 3,3'-diaminobenzidine

Adult mice were terminally anesthetized with ketamine (200 mg/kg) and xylazine (20 mg/kg) and their brains were immediately immersion-fixed in 4% paraformaldehyde (PFA) overnight at 4ºC. They were cryoprotected in 30% sucrose overnight at 4ºCand sectioned coronally at 40 μm thickness on a freezing microtome. Sections were rinsed three times (5 min each) in 0.1M phosphate-buffered saline (PBS); pretreated in 0.3% H_2O_2 for 30 min to block endogenous peroxidase activity; rinsed 2 times (5 min each) in PBS; blocked in 2% normal donkey serum (NDS, Jackson ImmunoResearch) in PBS for 30 minutes; incubated in 1:500 rabbit anti-collagen IV IgG [Abcam#19808] with 2% NDS and 0.3% TX in PBS for 2-3 days at 4ºC on a shaker; rinsed three times (10 min each) in PBS; incubated at room temperature for 90 min in 1:2,000 biotin-SP-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch); rinsed three times (10 min each) in PBS; incubated in the avidin-biotin-peroxidase complex (1:100 ABC; Vector Laboratories); rinsed 3 times (10 min each) in PBS; developed for 5 min in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; ISOPAC, Sigma-Alderich) with 0.01% H_2O_2 in PBS; rinsed in PBS; mounted out of water onto chromium/gelatin subbed slides, allowed to air-dry, coverslipped with Permount; and examined in bright field on a Zeiss AxioImager Z1.

Collagen IV/5-HT immunohistochemistry for confocal microscopy

At embryonic day 17 (E17), timed-pregnant dams were terminally anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg), and their uterus was dissected and kept in 0.1M PBS on ice. Embryos were removed from the uterus, decapitated, and their brains were dissected with fine forceps under a stereoscope. They were immersion-fixed in 4% PFA overnight at 4ºC, cryoprotected in 30% sucrose overnight at 4ºC, embedded in 20% gelatin (bloom 275), and sectioned coronally at 40 μm thickness on a freezing microtome.

Sections were then rinsed three times (5 min each) in 0.1M PBS; blocked in 2% NDS in PBS for 30 minutes; incubated in 1:250 rabbit anti-collagen IV IgG [Abcam#19808] and 1:400 goat anti-5-HT IgG [ImmunoStar #20079] with 2% NDS and 0.3% TX in PBS for 2-3 days at 4ºC on a shaker; rinsed three times (10 min each) in PBS; incubated at room temperature for 90 min in 1:1,000 AlexaFluor 488-donkey anti-rabbit IgG [Life Technologies] and 1:200 Cy3-donkey anti-goat IgG [Jackson ImmunoResearch] with 2% NDS in PBS; rinsed three times (10 min each) in PBS; mounted out of water onto chromium/gelatin subbed slides, allowed to air-dry, and coverslipped with the ProlongGold antifade medium with DAPI (Life Technologies). Confocal z-stacks (30-60 optical sections, 0.42 µm thick) were obtained with a 60X objective on the Olympus Fluoview 1000 Spectral Confocal system. Shark brains were dissected from the skulls with large rongeurs and processed the same way as the embryonic mouse brains. Adult mouse brains were processed under the same protocol.

Aim 2: Characterization of increased extracellular 5-HT levels on the expression of mPFC receptors

Animals

Adult C57BL/6 mice were purchased from Jackson Laboratories and housed individually on a 12:12 light-dark cycle with free access to water and food. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

Treatment Group

Mice were given an intraperitoneal injection of fluoxetine (an SSRI; 5 mg/kg) to increase extracellular 5-HT levels.

Tissue Collection and RNA Isolation

Twenty-four hours following injection, mice were terminally anesthetized using a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg). Their brains were dissected and put on ice for approximately three minutes. Brains were placed in a metal brain mold (Braintree Scientific) and 1 mm thick coronal slices of the rostral telencephalon were taken using sterile razor blades. The slices through the mPFC were mounted on glass slides and mPFC region was isolated under a stereoscope on dry ice using a brain punch (Stoelting Co. #57401). The punched slices imaged unstained on a Zeiss AxioImager Z1 with a 1X objective (Figure 2A). The total RNA was immediately extracted from the tissue punch with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA quality (the A260/A280 ratio) and concentration was measured with a NanoDrop spectrophotometer and the samples were stored at −80 °C until further processing. The quality of RNA (RIN) was measured with the TapeStation (Agilent Technologies) (Fig. 2B). From each sample, an estimated 20 mg of each RNA sample was reverse-transcribed to cDNA in an Eppendorf Mastercycler pro S using the iScript cDNA Kit (Bio-Rad) according to the manufacturer's instructions.

Quantitative RT-PCR Analysis

The RT-qPCR analysis was based on our published protocol (Hernandez & Janušonis, 2010). RT-PCR was run in a technical triplicate using SYBR green reagent (Qiagen). The expression of three serotonin receptors (5-HT1A, 5-HT2A, 5-HT4), and integrin β_3 (ITGB3) were quantified. Gene expression was normalized to three house-keeping reference genes (ubiquitin-C (UBC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and TATA box binding protein (TBP). The primers were designed in Primer 3 (Bio-Informatics Software) and are given in Table 1. The amplification was performed with the BioRad CFX96

thermocycler. The amplification conditions were as follows: 95 °C (3min); 39 cycles of 95 °C (15 s), 60 °C (15 s), 72 °C (10 s), 95 °C (10 s); and 60 °C (1 min). The efficiency of each amplification was calculated automatically by using a Mathematica (Wolfram Research, Inc.) program (Hernandez & Janušonis, 2010) based on a published algorithm (Tichopad, Dilger, Schwarz, & Pfaffl, 2003) with alterations. The obtained values were normalized to the geometric mean of the reference genes and multiplied by 1000 for convenience.

RESULTS

Mapping of microvasculature with confocal microscopy

First, the overall density of microvasculature was mapped in the adult mouse mPFC (Fig. 3). The capillaries appear uniformly distributed across the tissue, suggesting that there is homogeny in the density within gray matter. A three-dimensional reconstruction of adult mouse mPFC cells from z-stacks and Imaris software were used to provide a qualitative measure of the spatial association between brain vasculature and serotonergic varicosities (Fig. 4). However, the sheer density of 5-HT fibers fails to provide a clear view of the associations among varicosities and capillaries.

In order to better explore their spatial relationships, the next challenge was to better separate microvasculature and 5-HT varicosities. During development, serotonin fibers originating from the raphe nucleus invade the cortex. Upon initially reaching the cortex, rather than evenly innervating the region, 5-HT fibers form two bands. One band is located above the developing cortex, which at that point of development, is known as the cortical plate (CP; future cortical layers II-VI) and the second band resides in the marginal zone (MZ; future cortical layer I). Eventually, serotonergic fibers will be spread throughout all cortical layers, but at E17 in mice, 5-HT fibers are sparse within the CP (Janusonis, Gluncic, &

Rakic, 2004). Thus, the position of serotonergic fibers within the developing cortex of E17 mice presents a unique opportunity to analyze spatial composition of brain microvasculature and serotonergic fibers, unfettered by a heavy concentration of serotonergic fibers (Fig. 5). Single optical sections show 5-HT and capillaries to be closely associated with one another. It appears as though some of these 5-HT-positive profiles may be platelets situated within capillaries. Upon closer inspection, it appears as though these platelets are adhering close to the vascular walls and at times, appear to be escaping capillaries.

Swellshark images provide a chance to view serotonergic varicosities in an environment free from the presence of any potential transmigrated platelets. Since the swell shark has no peripheral 5-HT, studying a system with no platelet input allows a comparison of the differences and similarities between shark and rodent brains (Fig. 6). Comparing the confocal images of the two species leads to an interesting observation that the shark varicosities all seemed to display apparent axonal connections, while the mouse samples appeared to have 'loose varicosities' on occasion: a varicosity-like profile of which did not appear to have axonal connections to varicosities. Examining z-stacks from the shark brain did not reveal any 5-HT of which did not appear to be apart of a variscosity, though this observation was not apparent when viewing a single plane. These techniques do not provide a reliable way to distinguish between platelets and 5-HT fibers, yet they do point to the possibility that these free profiles are migrating platelets.

Effects of increased extracellular 5-HT on mRNA levels

RT-qPCR was performed for the mRNAs of 5-HT_{1a}R, 5-HT_{2a}R, 5-HT_4R, and ITGB3 in order to examine which receptors could be used as endogenous biological sensors of extracellular 5-HT levels in the mPFC. Statistical analyses were performed in SPSS 19

(IBM, Inc.). An independent samples t-test was used for each gene to compare the mean mRNA expressions for Fluoxetine-treated and saline control groups (Fig. 7). Fluoxetine treatment did not significantly alter mRNA levels for all but one of the tested receptors. Expression patterns were as follows: 5-$HT_{1a}R$ ($t(12)=-0.326$, $p=0.749$), 5-$HT_{2a}R$ ($t(14)=-0.403$, $p=0.693$), and 5-HT_4R ($t(14)=-0.891$, $p=0.388$). The mRNA expressions did not yield a significant effect, indicating that the transcription of the 5-HT_{1a}, 5-HT_{2a}, and 5-HT_4 receptors is not sensitive to acutely elevated 5-HT levels. These results contradict previous findings in our lab, where significant changes in mRNA expression resulted for these 5-HT receptors after acute SSRI administration (Chen, 2015). The mRNA that showed a significant change in expression was ITGB3 receptor ($t(12)=-2.330$, $p=0.038$) suggesting this as a potential indicator candidate.

DISCUSSION

It has not yet been investigated experimentally whether circulating blood platelets affect central 5-HT signaling. It is known that serotonergic varicosities are in close proximity to blood capillaries (Janušonis, 2014) and current research is continuing to challenge the impermeability of the BBB (Sotnikov et al., 2013), providing a basis to venture into projects that examine whether there is an exchange between the central and peripheral 5-HT systems. Our confocal data observed varicosity-like profiles in the mouse brain that may actually be transmigrated platelets. Since these curious varicosity-like profiles did not seem to be present within the swellshark brain, which does not contain any peripheral 5-HT (nor blood platelets), this may indicate that there are transmigrated platelets within the brain parenchyma that exhibit distinct (albeit subtle) profiles. However, this hypothesis needs to be tested, and we plan to add a simple measure to our analysis. We will selectively label

blood platelets *in vivo* with a staining reagent that carries a fluorescent (DyLight 649) tag (Emfret Analytics #X649) and use the Imaris system to automatically detect and count immunoreactive profiles. We expect that some fluorescently tagged platelets will be present in the brain parenchyma, indicating that peripheral 5-HT is able to cross the BBB and infiltrate the CNS. Whether or not these migrating platelets play a role in modulating central 5-HT signaling is yet another question currently left unaddressed.

While searching for a receptor sensitive to extracellular 5-HT changes, it was found that acutely increased 5-HT levels did not significantly affect the mRNA amounts of all studied 5-HT receptors in the adult mouse mPFC. Interestingly, the ITGB3 receptor was the most sensitive mRNA in the set. Integrins are cell adhesion molecules that facilitate the interaction between cell membranes and the extracellular matrix. They can be located in synapses, where they function to transduce signals between extracellular and intracellular domains (C.-S. Chan, Weeber, Kurup, Sweatt, & Davis, 2003). Recent evidence in human genetic studies suggest a role of ITGB3 in modulating 5-HT reuptake through its interactions with SERT (Whyte et al., 2014). One addiction study utilized immunoblotting and found that the ITBG3 subunit protein expression in the nucleus accumbens of adult mice was significantly increased 30 minutes after a cocaine challenge following chronic saline treatment (Wiggins, Pacchioni, & Kalivas, 2009). Results indicated that ITGB3 may be increasing actin cycling and modifying dendritic morphology in response to cocaine, which among its other actions, blocks 5-HT reuptake. This remarkably quick response in protein expression may be attributed to an acute sensitivity to increased extracellular 5-HT, which could explain our findings. The Wiggins findings suggest that the affinity of these receptors is increased by means of up-regulation or receptor modification, making it the best candidate

thus far for a functional monitor of changes in the levels of extracellular serotonin on a rapid time scale.

Our confocal data and observations provide a foundation for the ultimate objective of testing functional changes in central 5-HT signaling after direct blood platelet manipulation. In immunology, selective platelet depletion is a well-validated technique and is routinely used in mouse models. One feature of this technique is that platelets are short-lived and researchers can control the dynamics of their platelet counts by adjusting dose and administration (Fig. 8 and 9). Though we have not established that SSRIs have a rapid effect on the previously tested receptor expression in the adult mPFC using qRT-PCR, we seek to test more receptors as well as use more sensitive measures of mRNA expression. We plan to use future verified receptors as endogenous biological sensors to capture a central effect of depleting platelets. Using pharmalogical platelet depletion, we plan to assess changes in 5-HTR expressions in the mPFC using digital droplet PCR (ddPCR), a new technology available to our lab through the Biological NanoStructures Lab (California NanoSystems Institute, UCSB). With ddPCR, absolute gene quantification can be performed with a much higher quantitative sensitivity than qRT-PCR. This technique can detect very small changes in mRNA levels, so it may detect differences in expression of mPFC receptors that exhibit more subtle responses to altered 5-HT levels.

This paper has identified emerging evidence that platelets may be entering the brain parenchyma, using varicosity mapping in combination with confocal imaging. If platelets do reach and participate in CNS signaling, this means that extracellular 5-HT levels in the CNS not only depend on raphe signaling but also platelet transmigration. Platelets may well be a major underrepresented source of 5-HT that could change extracellular 5-HT levels in the

mPFC and thus affect an enormous range of behavioral processes. We aim to continue looking at platelet transmigration and its effect on mPFC 5-HT signaling in future projects, using the aforementioned procedures.

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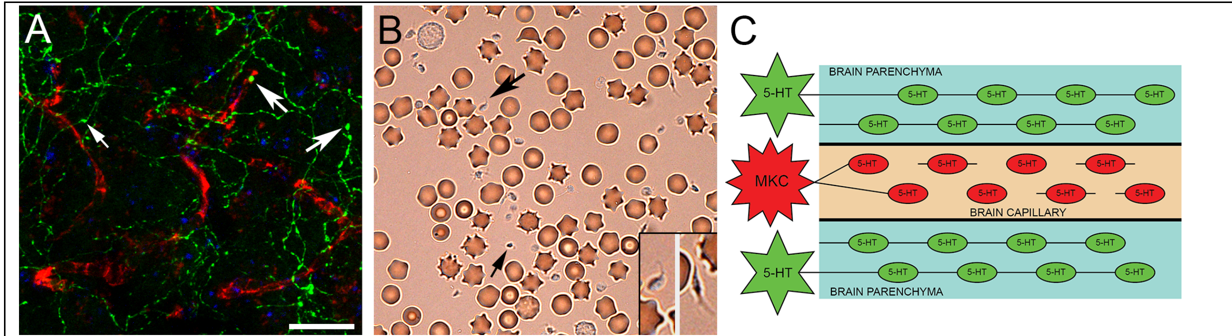

Figure 1. The similarity between serotonergic varicosities and blood platelets. **(A)** Confocal image of serotonergic fibers (green, immunohistochemistry), blood capillaries (red, rhodamine-conjugated dextran), and cell nuclei (blue, DAPI) in the mouse forebrain. **(B)** Bright-field image of a mouse blood sample (treated with EDTA to prevent coagulation). Some platelets are known to develop processes (Brecher & Cronkite, 1950). One such process is enlarged in the inset. These small processes are similar to some observed varicosities. **(A and B)** Arrows point to individual serotonergic varicosities and blood platelets. Scale= µm. **(C)** A schematic representation of 5-HT packets in the brain (green, serotonergic varicosities) and 5-HT packets in the blood (platelets, red) that coexist in close proximity. The star shapes represent serotonergic neurons in the raphe nuclei (5-HT) and a megakaryocyte in the bone marrow (MKC), both of which may be located remote distances from the 5-HT packets (not drawn to scale). Taken from Janusonis, 2014.

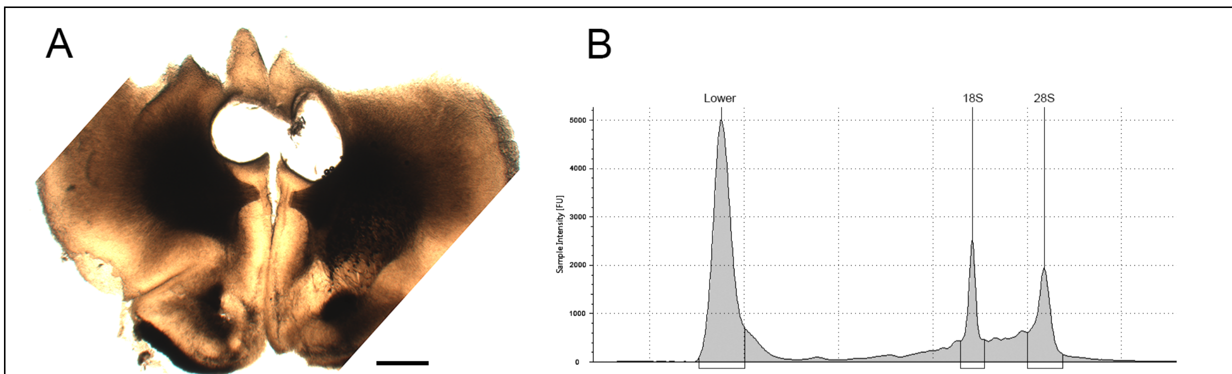

Figure 2. **(A)** Sample microdissection taken from the mPFC of an adult mouse using a 1.75mm gauge brain punch (Stoelting #57401) Imaged unstained on a Zeiss AxioImager Z1 with a 1X objective. Scale=1,000µm. **(B)** RNA analysis carried out using the Agilent 2200 TapeStation system. Representative electropherogram of total RNA from mPFC sample, the 18/28s peaks are annotated.

mRNA accession	Target mRNA	Forward primer (5'-3')	Reverse primer (5'-3')	bp
NM_008308	5-HT_{1A}R	CAGCGCGAGACA GATATTAC	CATCTGAGAGGA GCACTCAC	152
NM_172812	5-HT_{2A}R	TGACTGATTCCTC TCTGTGC	CCCCTCTCTTTGA GCTTCTA	196
XM_011246848 .1	5-HT_4R	GGAGATGTTCTGC CTGGTCC	CCAGCAGCCTCCC AACATTA	172
XM_011248766 .1	ITGB3	CTGCCGGAAGAA CTGTCACT	AGGCACAGTCAC AGTCGAAG	226
NM_013684.3 (reference)	TBP	AAGAGAGCCACG GACAACTG	TTCACATCACAGC TCCCCAC	183
NM_008084 (reference)	GAPDH	AATGTGTCCGTCG TGGATCTGA	AGTGTAGCCCAA GATGCCCTTC	117
NM_019639 (reference)	UBC	GATCTTTGCAGGC AAGCAGCT	TTCTCTATGGTGT CACTGGGCTC	174

Table 1. **The primer sequences for RT-qPCR**. Abbreviations: bp, amplicon length in base pairs; ; TBP, TATA box binding protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; UBC, Ubiquitin C

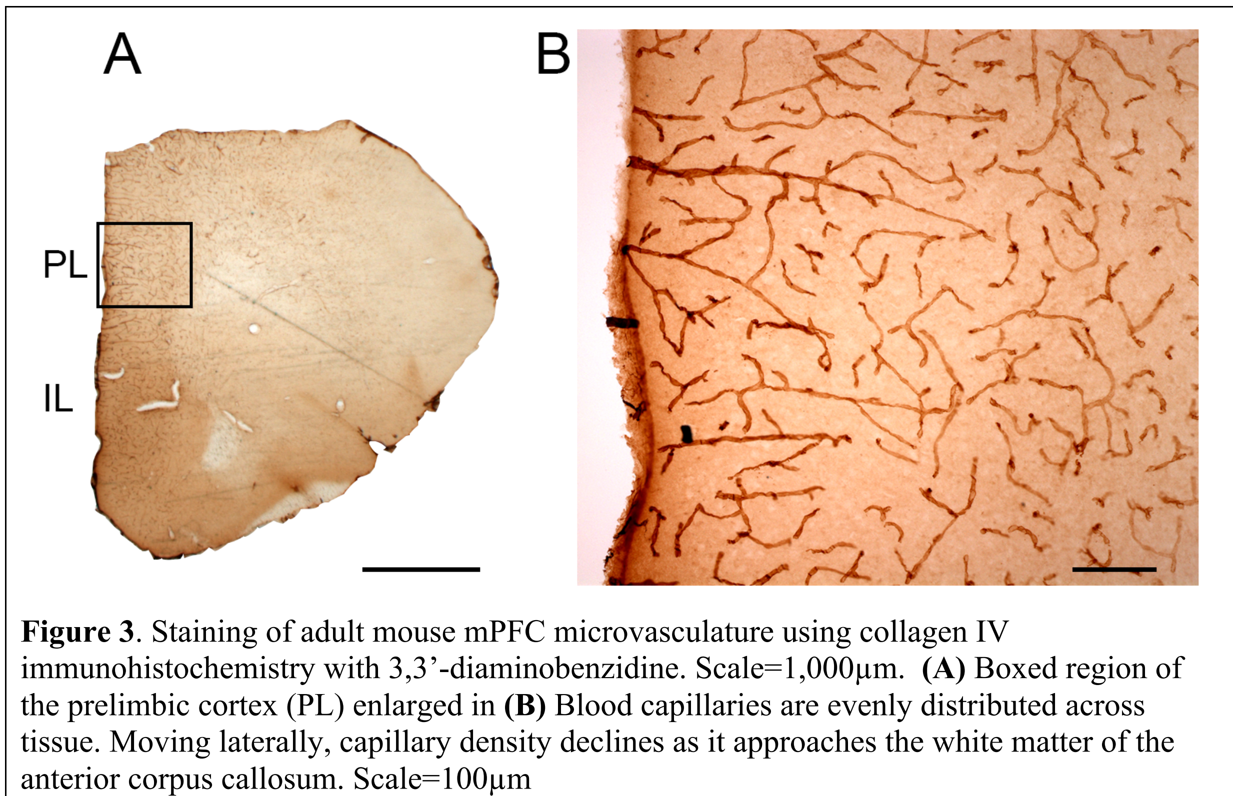

Figure 3. Staining of adult mouse mPFC microvasculature using collagen IV immunohistochemistry with 3,3'-diaminobenzidine. Scale=1,000µm. **(A)** Boxed region of the prelimbic cortex (PL) enlarged in **(B)** Blood capillaries are evenly distributed across tissue. Moving laterally, capillary density declines as it approaches the white matter of the anterior corpus callosum. Scale=100µm

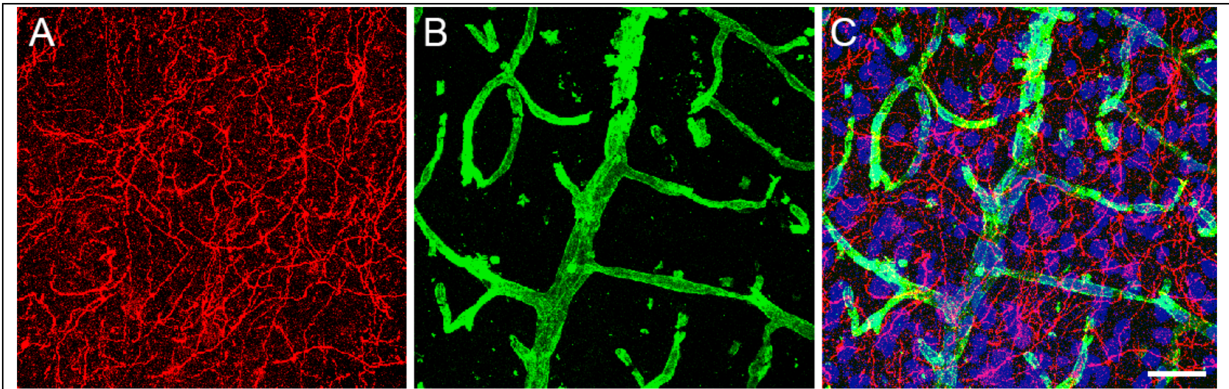

Figure 4. Confocal z-stacks showing **(A)** serotonergic fibers, **(B)** microvasculature (collagen IV), and **(C)** serotonergic fibers, microvasculature, and cell nuclei (blue) in the adult mouse mPFC. Note the studied processes rely on spatial relationship between capillaries and serotonergic fibers and **(C)** combined channels provide a crowded image with a very heavy dense meshwork of serotonergic fibers superimposed on capillaries. Scale=30μm.

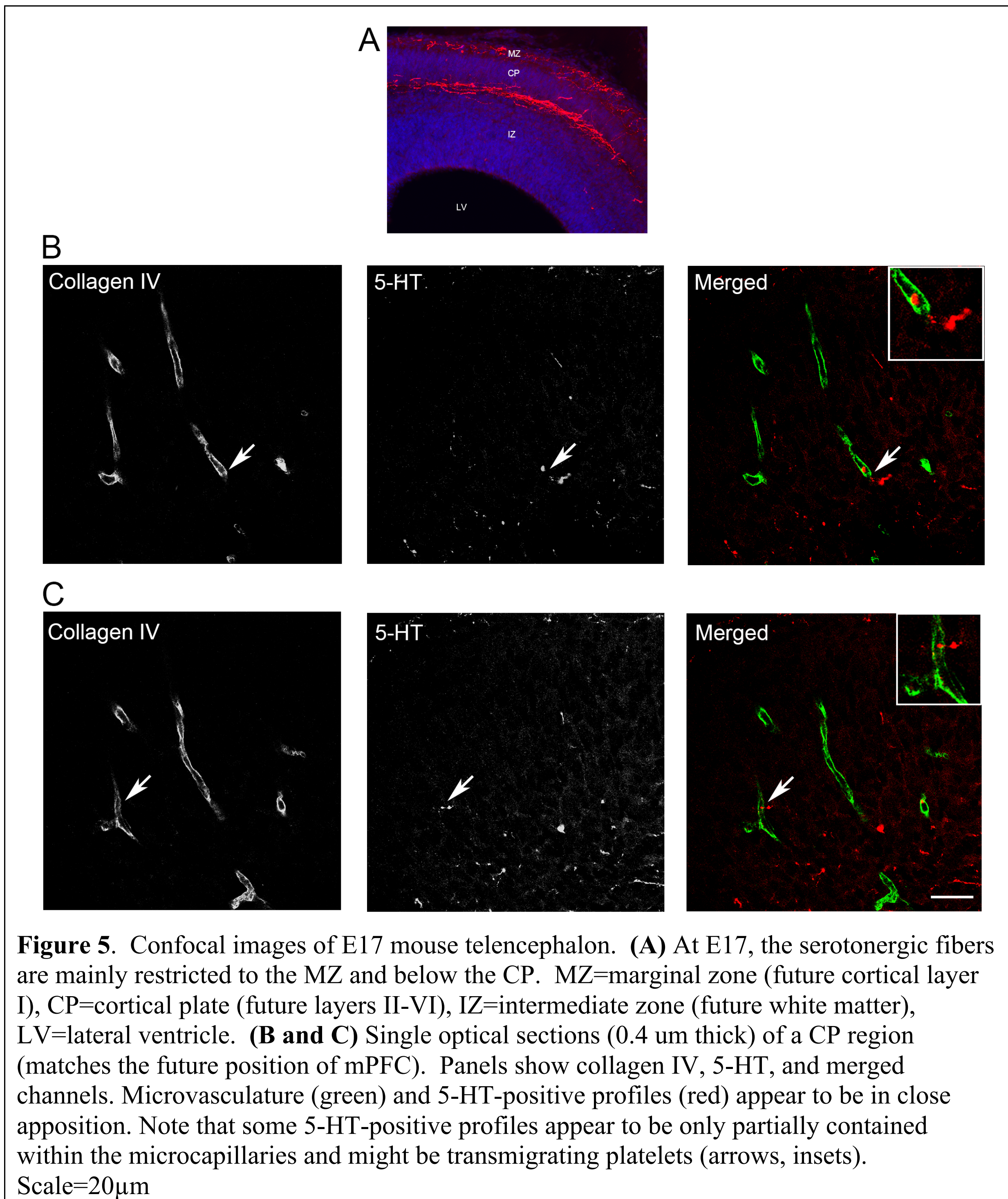

Figure 5. Confocal images of E17 mouse telencephalon. **(A)** At E17, the serotonergic fibers are mainly restricted to the MZ and below the CP. MZ=marginal zone (future cortical layer I), CP=cortical plate (future layers II-VI), IZ=intermediate zone (future white matter), LV=lateral ventricle. **(B and C)** Single optical sections (0.4 um thick) of a CP region (matches the future position of mPFC). Panels show collagen IV, 5-HT, and merged channels. Microvasculature (green) and 5-HT-positive profiles (red) appear to be in close apposition. Note that some 5-HT-positive profiles appear to be only partially contained within the microcapillaries and might be transmigrating platelets (arrows, insets). Scale=20µm

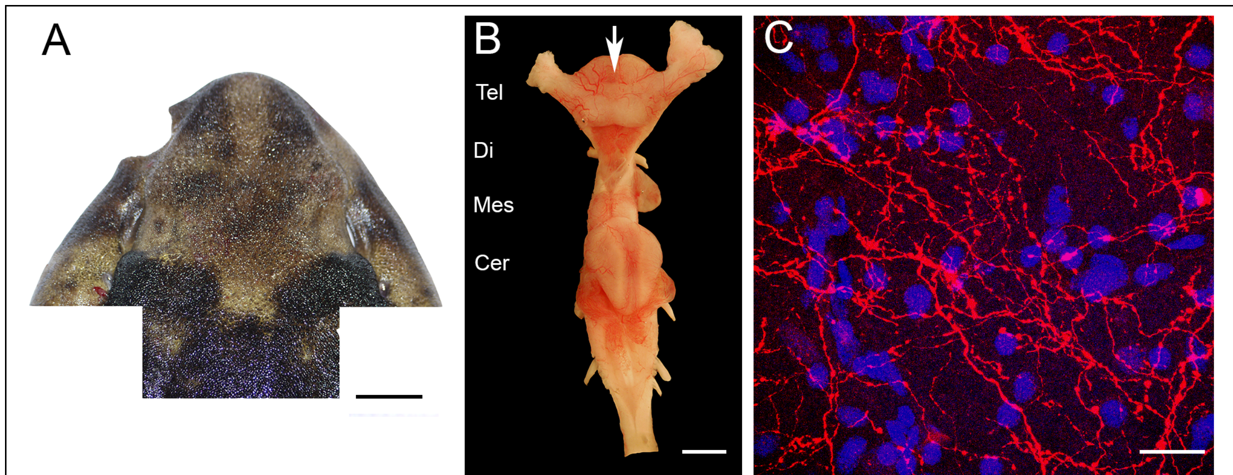

Figure 6. (A) Freshly caught swellshark **(B)** telencephalon (arrow) was dissected (scale=5mm) and **(C)** confocal imaging of serotonergic varicosities (red) and cell nuclei (blue). Note that swellsharks do not carry blood platelets nor do they have any 5-HT in circulation, thus **(C)** offer a central 5-HT system that is not confounded by peripheral 5-HT. Fibers are notably strong, with no apparent platelet-like profiles present when viewed within 3D z-stacks. Scale=20μm

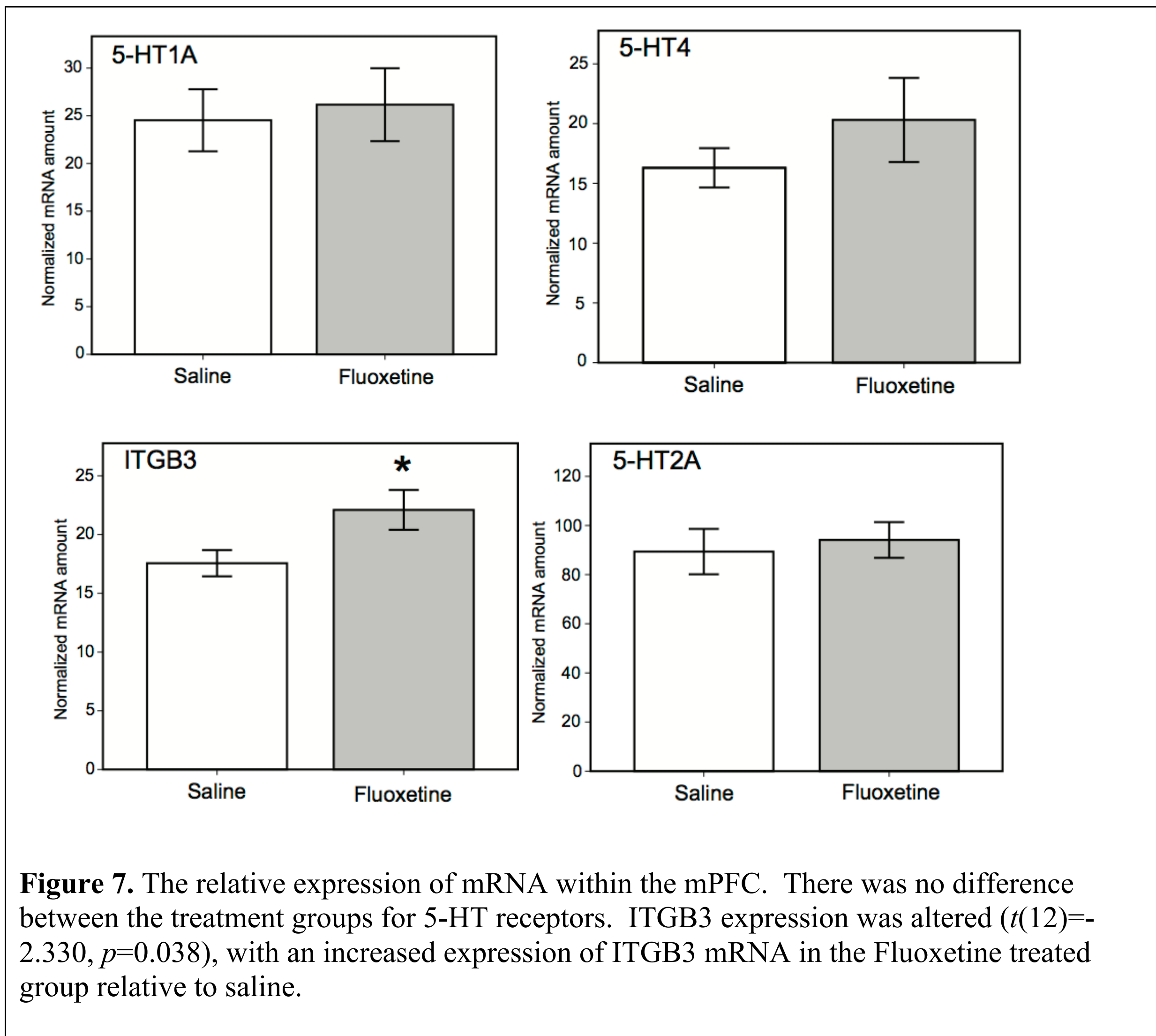

Figure 7. The relative expression of mRNA within the mPFC. There was no difference between the treatment groups for 5-HT receptors. ITGB3 expression was altered ($t(12)=-2.330$, $p=0.038$), with an increased expression of ITGB3 mRNA in the Fluoxetine treated group relative to saline.

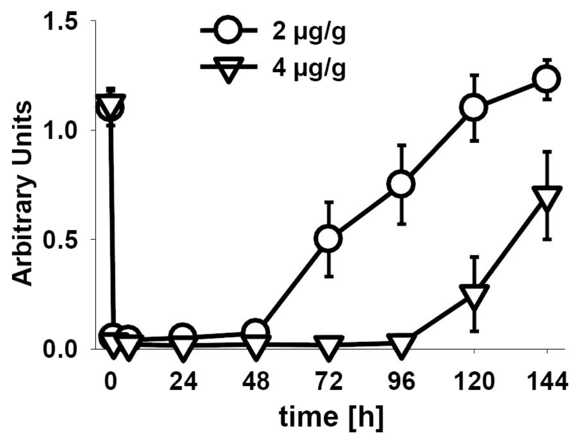

Figure 8. The platelet count following a single injection (2ug per g body weight) of a mixture of purified rat monoclonal antibodies against mouse GP1bα (CD42b) (data from the Emfret Analytics). This mixture reduces platelet count by over 95% within 60 minutes after treatment and remain knocked down for 48 hours.

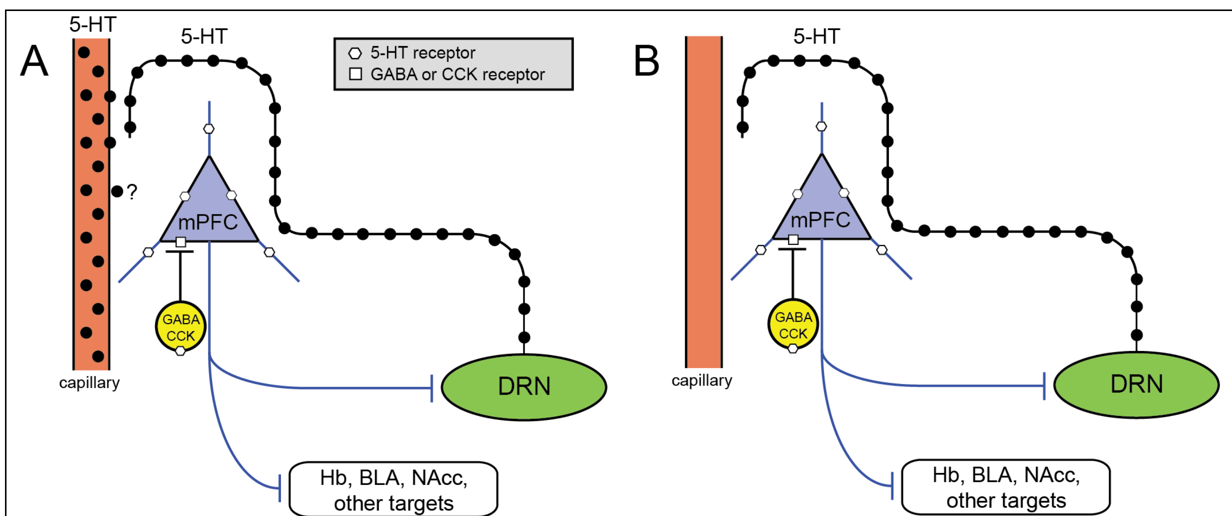

Figure 9. **(A)** mPFC pyramidal neurons (blue triangle) and GABAergic interneurons (yellow circle) express 5-HT receptors that monitor extracellular 5-HT. All 5-HT present in the brain is thought to be released from serotonergic varicosities (black connected circles) that are located on axons projecting mainly from the dorsal raphe nucleus (DRN; green oval) However, the mPFC may contain an alternative 5-HT source: blood platelets (black, unconnected circles). Platelets bear a resemblance to serotonergic varicosities and may escape capillaries to enter the brain parenchyma. **(B)** If this hypothesis is correct, selective platelet depletion should have an effect on 5-HT signaling in the mPFC.