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Janušonis, S

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Serotonin dynamics in and around the central nervous system: Is autism solvable without fundamental insights?



Skirmantas Janušonis*

Department of Psychological and Brain Sciences, University of California, Santa Barbara, CA 93106-9660, USA

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ABSTRACT

Altered serotonin (5-hydroxytryptamine, 5-HT) signaling has been implicated in some developmental abnormalities of autism spectrum disorder (ASD). However, the presumed role of 5-HT in ASD raises new questions in fundamental neuroscience. Specifically, it is not clear if the current piecemeal approach to 5-HT signaling in the mammalian body is effective and whether new conceptual approaches may be required. This review briefly discusses 5-HT production and circulation in the central nervous system and outside of it, especially with regard to ASD, and proposes a more encompassing approach that questions the utility of the “neurotransmitter” concept. It then introduces the idea of a generalized 5-HT packet that may offer insights into possible links between serotonergic varicosities and blood platelets. These approaches have theoretical significance, but they are also well positioned to advance our understanding of some long-standing problems in autism research.

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1. Introduction: Autism and fundamental neuroscience

The problem of autism is a proverbial rabbit hole that leads to problems in fundamental neuroscience. Over the past decade, autism spectrum disorder (ASD) has become an ultimate Rorschach test in neurobiology, and the pathologies that have been associated with ASD now cover a vast array of neurobiological processes and brain structures (Rodier, 2002; Courchesne et al., 2007; Amaral et al., 2008; Fatemi et al., 2012; Gadad et al., 2013; Gesundheit et al., 2013; Parellada et al., 2014; Banerjee et al., 2014). Likewise, massive genetic studies have yielded enormous lists of potentially relevant but weakly associated genes (Anney et al., 2010; Betancur, 2011; Lee et al., 2013), the fundamental or practical value of which has yet to be demonstrated. If ASD is defined as a specific applied problem, fundamental neurobiology thus far has contributed surprisingly little to its solution.

The current situation calls for an honest reassessment of where we truly are. A contemporary scientist is an odd cross between an objective observer unbound by the here-and-now and a merchant who needs to sell his/her goods to the public. This can and does work in many science fields, but autism research has been heavily biased toward the latter. Efforts to serve families with autistic children have a noble goal, but they are best left to applied fields seeking

to improve interventions, therapies, and treatments. Beyond that, ASD may be simply unsolvable without a much deeper understanding of the key building elements that make this condition possible. This may require major improvements in fundamental neuroscience and may contradict the public's intuition of what is important.

At present an unsettling possibility remains that ASD may be a kind of “limping syndrome” that can be easily observed and approached therapeutically, but that cannot be rigorously studied as a single neurobiological entity. A more optimistic view is that ASD is a solvable problem, but fundamental neuroscience is not ready for it (to paraphrase Paul Erdős' comments on the $3n + 1$ conjecture). ASD is likely to be the tip of a large iceberg, but focusing on this visible tip may be a shortsighted approach. Instead, a few fundamental problems have to be addressed directly and immediately. The most obvious one is how normal brains align their neural activities in social interactions. Another one is the interplay between the cognitive and motor systems in the brain (which may lead to major revisions in our current understanding of the basal ganglia and the cerebellum). This brief review focuses on yet another deep problem that thus far has attracted too little attention: the integrated dynamics of serotonin (5-hydroxytryptamine, 5-HT) in the mammalian body.

2. Serotonin in autism

A large number of studies have shown that ethnically diverse groups of autistic individuals have elevated mean 5-HT levels in

* Tel.: +1 805 893 6032; fax: +1 805 893 4303.

E-mail address: skirmantas.janusonis@psych.ucsb.edu

blood platelets (Schain and Freedman, 1961; Hanley et al., 1977; Anderson et al., 1987b, 1990, 2002b; Cook, 1996; McBride et al., 1998; Coutinho et al., 2004, 2007; Mulder et al., 2004; Hranilovic et al., 2007, 2008; Melke et al., 2008; Tordjman et al., 2013). This phenomenon, known as the blood or platelet hyperserotonemia of autism, is considered to be one of the most well-replicated findings in biological psychiatry (Anderson, 2002).

The biological causes of the platelet hyperserotonemia remain unknown (Janušonis, 2008), despite the fact that this observation is over half a century old. This surprisingly long-lasting ignorance can be explained, in part, by the fact that the blood is a relatively foreign system to most neuroscientists. Even though the brain has a very dense capillary network (Duvernoy et al., 1981), the anatomical basis for functional MRI imaging, the blood is typically assumed to be on the other side of the blood-brain barrier (BBB) and therefore not immediately relevant to most neurobiological experimentation. This conveniently ignores the fact that the BBB is built after the brain comes into existence during development, and that it is actually a shorthand for a number of barriers that emerge at different developmental times (Wenzel and Felgenhauer, 1976; Virgintino et al., 2004; Ge et al., 2005; Daneman et al., 2010; Liddelow et al., 2013). In the human brain, tight junctions develop prenatally (Mollgard and Saunders, 1986; Bell et al., 1991; Virgintino et al., 2004). In the adult brain, the BBB remains a flexible entity and is better comparable to a country's immigration policies than to the Great Wall of China. The BBB can be dynamically modulated by various environmental factors, such as drugs, stress, and others (Sharma, 2004a). Importantly, the BBB is likely to be affected in ASD (Theoharides and Zhang, 2011; Noriega and Savelkoul, 2014).

Rodent models of hyperserotonemia have found associations between blood 5-HT levels and autistic-like behaviors (Kahne et al., 2002). Further studies have shown related alterations in the oxytocin system (Whitaker-Azmitia, 2005; McNamara et al., 2008; Madden and Zup, 2014). One caveat in these models is that they use 5-methoxytryptamine (5-MT), a non-selective agonist on several 5-HT receptors. While this non-selectivity correctly mimics the action of 5-HT itself, it is not clear if 5-MT is taken up by blood platelets. Currently, there is no evidence that in autism 5-HT levels are also elevated in platelet-free plasma, and some studies have reported the opposite effect (Spivak et al., 2004).

We have shown that a commonly used inbred mouse strain, C57BL/6, shows transiently accelerated brain growth with respect to another inbred mouse strain, BALB/c (Flood et al., 2012). A key difference between these strains is that a polymorphism in the *Tph2* gene leads to a relatively higher 5-HT synthesis rate in the brain of the C57BL/6 strain (Zhang et al., 2004). This transiently accelerated growth is similar in magnitude to that observed in autistic brains (Courchesne et al., 2003). Interestingly, the C57BL/6 strain also shows blood hyperserotonemia with respect to the BALB/c strain, which persists into adulthood (Flood et al., 2012).

Considerably less is known about 5-HT alterations in the brains of autistic individuals. One study has found an altered developmental dynamic of 5-HT synthesis capacity in autistic brains (Chugani et al., 1999), but these results have yet to be independently replicated. A recent study has shown that in autism the density of serotonergic fibers in the cortex is significantly higher than in normally developing brains (Azmitia et al., 2011). Again, a replication of these results in a larger set of brains is important, because the immunohistochemical 5-HT signal is often sensitive to the intensity and length of fixation, a factor that is difficult to control in autopsied human brains. If this finding holds up, it will become one of the most important discoveries in autism research.

In summary, there is little doubt that 5-HT signaling is affected in ASD, perhaps very early in development, but the specifics (or even directions) of these alterations in the central nervous system

(CNS) remain elusive. In this regard, the platelet hyperserotonemia of autism is a remarkably robust finding.

3. A traditionalist view: Two 5-HT systems

In order to understand the dynamics of 5-HT in the mammalian body, one can start with an observation that there are two principal sites of 5-HT production: the gastrointestinal system and the brain. Both systems have a tubular organization, which may or may not be coincidental (Veeman et al., 2010). In both systems, 5-HT is synthesized from L-tryptophan, an amino acid. In both systems, 5-HT is typically removed by converting it to 5-hydroxyindoleacetic acid (5-HIAA). Tryptophan can cross the BBB, but its entry into the CNS is limited by the competition among several neutral amino acids (tryptophan is one of them). In contrast, the BBB is generally thought to be impermeable to 5-HT, which implies two virtually independent 5-HT pools in the body: one inside and the other outside the CNS. It is important to note that the non-CNS 5-HT, when released into the general blood circulation, flows through the CNS in the immediate vicinity of neurons, but it is assumed to be unable to escape into the brain parenchyma.

In the CNS, 5-HT is synthesized by serotonergic neurons, most of which are located in the raphe nuclei of the brainstem. In this system, the enzyme that converts tryptophan into 5-hydroxytryptophan (5-HTP), the immediate precursor of 5-HT, is tryptophan hydroxylase 2 (*Tph2*) (Walther et al., 2003). In the CNS, 5-HT can be detected by (perhaps all) neurons and glial cells (astrocytes, oligodendrocytes, and microglia) (Cohen et al., 1999; Whitaker-Azmitia, 2001; Millan et al., 2008), as well as by endothelial cells, as described later. Serotonergic fibers (axons), originating in the raphe nuclei, spread throughout the brain, which becomes virtually embedded in their meshwork. Even though it is typically assumed that 5-HT is released from serotonergic fibers "diffusely," 5-HT signaling among neurons may occur through conventional synapses (Papadopoulos et al., 1987; Parnavelas and Papadopoulos, 1989). Extracellular 5-HT is pumped by the 5-HT transporter (SERT) back into serotonergic fibers, where the 5-HT may be recycled. The extracellular 5-HT concentration in the brain is low and in the rat rostral raphe nuclei may not usually exceed 2–8 nM. This concentration may not be high enough to activate 5-HT_{1A} autoreceptors unless 5-HT levels become excessive (Adell et al., 2002). It has been reported that in development some neurons may express tryptophan hydroxylase 1 (*Tph1*) (Nakamura et al., 2006; Manjarrez-Gutierrez et al., 2012), but these observations may need further validation (Hale et al., 2011).

Developing thalamocortical neurons (and some other non-serotonergic neurons) can transiently express SERT and take up 5-HT, even though they themselves do not synthesize 5-HT (Lebrand et al., 1996, 1998). The exact pattern of the transient SERT expression shows species-specific variation (Lebrand et al., 2006).

In the CNS, 5-HT is converted to 5-HIAA that enters the cerebrospinal fluid (CSF) and can be measured in lumbar puncture samples. The concentration of 5-HIAA in the human CSF has been estimated to be around 122 nM (Narayan et al., 1993). These 5-HIAA levels can be used to indirectly assess 5-HT function in the normal and autistic CNS (Anderson et al., 1988; Narayan et al., 1993). It has been suggested that CSF 5-HT levels, when analyzed with great care to minimize blood contamination, may provide a more direct and accurate measure of extracellular 5-HT in the CNS (Anderson et al., 2002a). The concentration of 5-HT in the CSF of *Macaca mulatta*, a non-human primate, has been estimated to be around 87 ng/L (Anderson et al., 2002a).

Outside the CNS, most 5-HT is synthesized by gut enterochromaffin (EC) cells, with some contribution from neurons in the enteric nervous system (ENS). Over 95% of the body 5-HT is in the

gut and over 90% of this 5-HT is stored in EC cells that are distributed in the enteric epithelium from the stomach through the colon (Gershon, 2004). The enzyme that converts tryptophan into 5-HTP in EC cells is tryptophan hydroxylase 1 (Tph1), but ENS neurons use Tph2, just like neurons in the CNS (Walther et al., 2003; Gershon and Tack, 2007). Functionally, the 5-HT produced in the gut plays two distinct roles.

First, gut 5-HT serves as a signaling molecule (or a “neurotransmitter”) in the ENS (Gershon, 2003), which may contain as many neurons as the spinal cord (Gershon, 2004). Extracellular 5-HT is taken up by neuronal and non-neuronal cells of the gut that express SERT (Gershon, 2003, 2004). SERT may be important in intestinal immune and inflammatory responses, since abnormal SERT function may increase the severity of immune-mediated colitis (Bischoff et al., 2009).

Second, gut 5-HT enters the systemic blood circulation, where most of this 5-HT is rapidly cleared by the liver, the lungs, and other organs (Thomas and Vane, 1967; Anderson et al., 1987c). Some of the remaining 5-HT is taken up and stored by blood platelets that express SERT (Lesch et al., 1993) but do not synthesize 5-HT themselves. Blood platelets are an essential component of the blood, where they play important roles in blood clotting and the regulation of vascular tone. They are small (around 1.5–3.0 μm in diameter) protoplasmic disks that have no nucleus. In hematopoiesis, they split off from long processes of large (up to 60 μm in diameter), polyploid megakaryocytes in the bone marrow. Blood platelets are short-lived; their half-life has been estimated to be only 4–6 days (Heysse, 1961; Stuart et al., 1975). Since 5-HT is constantly added to and cleared from the systemic blood circulation, the concentration of free 5-HT in the blood plasma varies in different parts of the peripheral 5-HT system. In practice, free plasma 5-HT is usually measured in the distal venous blood, after the liver and the lungs have removed most of the 5-HT released from the gut (Anderson et al., 1987c). This concentration is low and has been estimated to be 304 ng/L (Anderson et al., 1987a) and 0.77 nM (Beck et al., 1993). Because of considerable technical challenges, its actual value remains unknown but it is undoubtedly below 1000 ng/L (Anderson, 2007). Since the levels of 5-HT in blood platelets are a few orders of magnitude higher, whole-blood 5-HT levels are very similar to the levels of the 5-HT sequestered in blood platelets. In normal subjects, these whole-blood 5-HT levels have been estimated to be around 187–297 $\mu\text{g/L}$ (McBride et al., 1998) and 0.4 μM (Melke et al., 2008); other studies have reported comparable concentrations (reviewed in Janušonis, 2008).

Outside the CNS, 5-HT is converted to 5-HIAA that can be detected in the urine. Expressed per mg creatinine (in order to normalize for the body surface area and some other variables), urinary 5-HIAA concentration has been estimated to be 3.5 $\mu\text{g/mg}$ creatinine in normal subjects (Minderer et al., 1987). The urine also contains 5-HT; urinary 5-HT levels have been found to be around 0.1 $\mu\text{g/mg}$ creatinine in normal subjects (Anderson et al., 1989).

A reader interested in more information about autistic alterations of 5-HT within and outside the CNS is referred to a recent review (Yang et al., 2014).

4. Quantitative modeling

The platelet hyperserotonemia of autism raises interesting questions because blood platelets survive only a few days and are constantly replaced with new platelets. This suggests that the process that overloads platelets with 5-HT continues to be active years after the CNS has developed. Some of these same molecular factors may play key roles in the development of the autistic brain, well before ASD is diagnosed. In this regard, blood platelets may serve as a window into the unobservable past of the autistic brain.

At present, only two quantitative models of platelet 5-HT are available (Anderson et al., 1987c; Janušonis, 2008). This situation is surprising because multi-variable systems with feedback loops are not readily amenable to intuitive reasoning. If the reader is not convinced, he/she is challenged to predict the trajectory of the following recursive process with a single variable: $x_{n+1} = Ax_n(1 - x_n)$, where $A = 2.0$ or $A = 3.5$. Despite the obvious importance of dynamical reasoning in neurobiology and biology in general, many system-level experimental interpretations still rely on “common sense,” which is a dangerous approach when one deals with dynamically interacting variables (Janušonis, 2012a,b; Kumar et al., 2013). Specifically, many studies have investigated SERT function in the platelet hyperserotonemia of autism (some of them reviewed in Janušonis, 2008). An increase in SERT function may reduce the amount of 5-HT released from the gut into the general blood circulation, but increase 5-HT uptake by platelets. The final platelet 5-HT levels will depend on the combined dynamics and numerical values of the variables participating in the process.

5. A generalization: 5-HT is a generic signal carrier that is also present in the CNS

Science is produced by the human brain whose operation heavily relies on implicit Bayesian associations and assumptions. Because of these inherent biases, it matters greatly what concepts and names we use to think about the CNS. Perhaps one of the most misleading terms in neuroscience is that of “neurotransmitter.” Many, if not most, “neurotransmitters” are generic signaling molecules present throughout the invertebrate and vertebrate bodies that simply have not been left out of their nervous systems. Indeed, 5-HT is present as a signaling molecule in all studied multicellular animals (metazoans) (Hay-Schmidt, 2000; Kass-Simon and Pierobon, 2007; Mayorova and Kosevich, 2013), including those that do not have a nervous system (Czaker, 2006). Likewise, 5-HT and 5-HT receptors control early embryogenesis, such as gastrulation, before the nervous system develops (Buznikov, 1984; Emanuelsson et al., 1988; Buznikov et al., 2001, 2005; Levin et al., 2006). Recently, a study has detected the mRNA transcripts of nearly all 5-HT receptors in the avian germ cells or early embryos (Stepinska et al., 2014), and 5-HT receptors have been found in the hamster and human sperm cells (Fujinoki, 2011; Jimenez-Trejo et al., 2012).

Not surprisingly, 5-HT and 5-HT receptors are also present throughout the mammalian body, where they control various functions in craniofacial development (Shuey et al., 1992; Moiseiwitsch and Lauder, 1995), the gastrointestinal system (Gershon and Tack, 2007; Sanger, 2008; Bertrand and Bertrand, 2010), the heart (Bach et al., 2001; Lezoualc’h et al., 2007; Derangeon et al., 2010), the blood (Anderson, 2002), and many other organ systems (Berger et al., 2009). Recently, production of 5-HT by the placenta has been shown to be an important factor in the fetal development of the forebrain (Bonnin et al., 2011; Bonnin and Levitt, 2011).

At first glance, the pervasive distribution of 5-HT as an information-carrier in the animal body may appear irrelevant to understanding autism, which by definition is a brain phenomenon specific to the human and (perhaps) some other mammalian species. This approach is CNS-centric, naïve, and undermines the very foundation upon which we should build. With a few exceptions discussed in the next section, there is nothing special about 5-HT in the brain. Many cell communities, including brain cell communities, depend on this modified amino acid to encode information. This may explain why autistic behavioral symptoms are often accompanied not only by elevated 5-HT levels in blood platelets, but also by gastrointestinal dysfunction (Hsiao, 2014) and perhaps other “peripheral” abnormalities. In particular, global

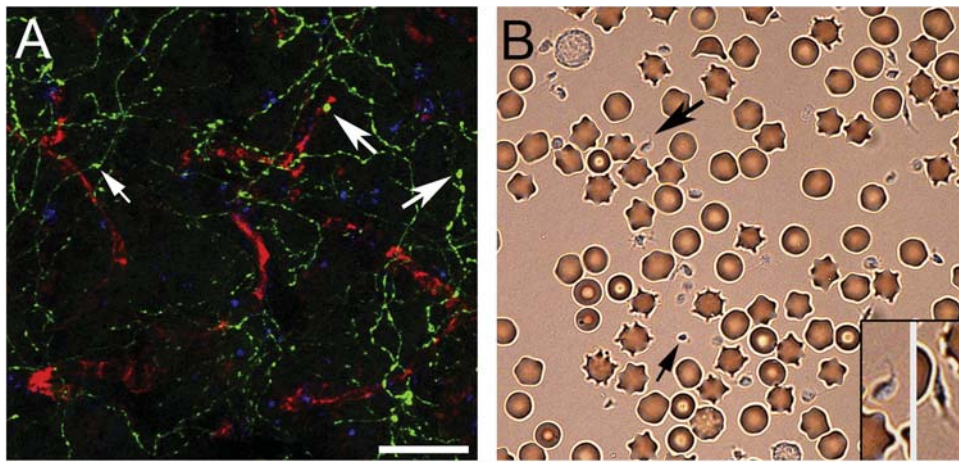


Fig. 1. The similarity between 5-HT packets in the brain (serotonergic varicosities) and 5-HT packets in the blood (blood platelets). (A) A confocal image of serotonergic fibers (green, immunohistochemistry), blood capillaries (red, rhodamine-conjugated dextran), and cell nuclei (blue, DAPI) in the mouse forebrain. (B) A bright-field image of a mouse blood sample (treated with EDTA to prevent coagulation). Note that some platelets have processes (enlarged in the inset). (A and B) The arrows point to individual serotonergic varicosities and blood platelets. The scale is the same in both panels (scale bar = 20 μm). Since the image in B is not confocal, some platelets may be slightly out of focus and appear bigger. The densities of serotonergic varicosities and platelets are not comparable because the blood smear (B) does not represent the same tissue volume that is shown in A. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

“peripheral” G protein signaling may be affected in ASD (Jacobson et al., 2014), which is an important observation in light of the transcriptional associations among G protein-coupled receptors in the human brain (Janušonis, 2014).

6. A new concept: The generalized 5-HT packet

The previous section suggests that we would benefit from a more encompassing view of 5-HT signaling and that it could offer fresh insights into the biology of ASD. I now focus on one aspect of this general idea and suggest that we consider a new conceptual entity, the generalized “5-HT packet.”

Serotonergic varicosities in the CNS and platelets in the blood share a number of obvious features (Fig. 1). Both have roughly the same size (Benzekhroufa et al., 2009; McGarry et al., 2010) and carry no nuclei. In the case of serotonergic varicosities, the cell body that produced them (a raphe neuron) can be located a long distance away, in the lower brainstem. Any randomly chosen varicosity is presumed to be attached to a serotonergic cell body, but this has not been tested experimentally. Likewise, the full trajectory of a single serotonergic axon has never been traced in the adult brain. Blood platelets are also produced by cells that are located a large distance away, the megakaryocytes in the bone marrow. Platelets do not remain attached to them, but can develop processes, some impressively long, in EDTA-treated blood smears (Brecher and Cronkite, 1950). At least superficially, a platelet with a process may resemble a serotonergic varicosity that uses a thin axonal tether to attach to the next varicosity (Fig. 1).

The similarity between serotonergic varicosities and blood platelets extends to the molecular level. Both express the SERT coded by the same gene (Lesch et al., 1993) and both use it to take up 5-HT from the extracellular space. Human platelets express several 5-HT receptors (Amisten et al., 2008), among which the 5-HT_{2A} receptor has been particularly well studied, including its possible association with ASD (Hranilovic et al., 2009). Molecular similarities between varicosities and platelets run even deeper and, interestingly, have been known for decades (Sneddon, 1973; Stahl, 1977).

The current understanding is that serotonergic varicosities and blood platelets reside on the two opposite sides of the BBB and cannot interact. It appears to be a reasonable assumption considering that even free 5-HT molecules may not cross the BBB. However,

the impermeability of the BBB to 5-HT has recently been challenged in a study that has shown that elevated brain 5-HT levels can cause a significant increase in blood 5-HT levels (Nakatani et al., 2008). Also, cells much larger than platelets can get out of brain blood capillaries and reach neurons normally protected by the BBB. Thus far, bone marrow-derived monocytes have been particularly well studied in this regard (Anderson et al., 2004; Wu et al., 2006; Lampron et al., 2013). While this transmigration is typically injury- or disease-dependent and requires cell-adhesion interactions between the migrating and endothelial cells in the vascular wall (Anderson et al., 2004), it raises a question of what else can cross the BBB under favorable conditions. It should be noted that the permeability of the BBB can be controlled by 5-HT acting on 5-HT₂ receptors in the CNS microvasculature, and that either luminal or abluminal application of 5-HT to blood capillaries can increase BBB permeability (Sharma, 2004b). Since these capillaries separate the two types of 5-HT packet (Fig. 2), further questions can be raised about the presumed independence among the components of this system.

Serotonergic varicosities tend to be located close to blood capillaries. It has been estimated that in the rat frontoparietal cortex around 10% of serotonergic varicosities are directly associated with microvasculature. On average, these varicosities are located only 1 μm away from a microvessel, the distance that is smaller than the size of a typical platelet (Cohen et al., 1996). Further, the concentrations of serotonergic varicosities in the brain and platelets in the blood are comparable. The density of serotonergic varicosities in the rat cerebral cortex has been estimated to be around 6,000,000/mm³ cortical tissue (Jacobs and Azmitia, 1992), and the density of platelets in the mouse blood is around 300,000–1,000,000/mm³ blood (McGarry et al., 2010). Considering both of these numbers are only approximate, they are in relatively good agreement. The concentrations of free 5-HT on the inside and outside of brain capillaries may also be comparable (Anderson et al., 1987b; Beck et al., 1993; Adell et al., 2002; Anderson, 2007), but these measurements pose major technical challenges and should be treated with caution.

The exchange between serotonergic varicosities and blood platelets, direct or indirect, is currently hypothetical and has to be investigated experimentally. However, it is becoming clear that the current textbook version of the brain “serotonin system” gives a warped picture that does not facilitate deep conceptual thinking.

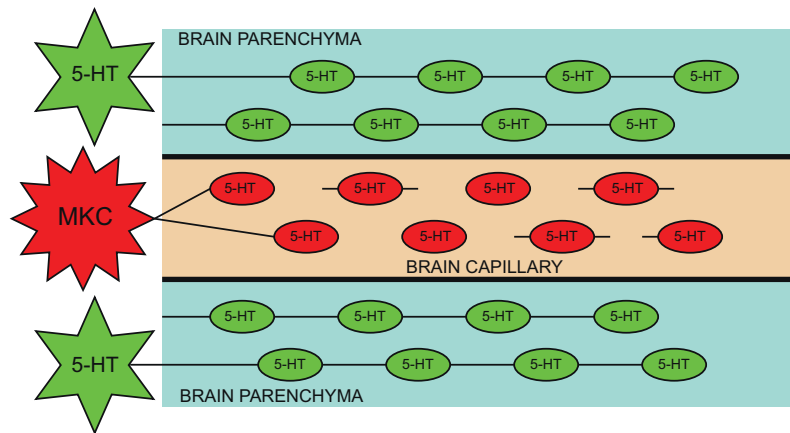


Fig. 2. A schematic representation of 5-HT packets in the brain (serotonergic varicosities, green) and 5-HT packets in the blood (blood platelets, red) that coexist in close physical proximity. It is not known if platelets *in situ* ever develop processes, but they might. The star shapes represent serotonergic neurons in the raphe nuclei (5-HT) and a megakaryocyte in the bone marrow (MKC), both of which may be located far away from the 5-HT packets (which are not drawn to scale). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In a twist of irony, the problem of autism might be a gift to fundamental neuroscience that may be forced to reconsider its most entrenched dogmas. One can hope that in a short time it will be able to offer a generous payback.

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