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REVIEW

Techniques for in vivo serotonin detection in the brain: State of the art

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Abstract

Neuronal circuits in the brain that utilize the neurotransmitter serotonin are essential to the regulation of mood and emotional expression. Disruptions in serotonin signaling underlie neuropsychiatric conditions such as depression and anxiety. However, the cellular mechanisms that regulate serotonergic signaling in the brain in healthy and diseased states remain to be better understood. In particular, as more is learned about serotonin in the brain, we recognize an urgent need to develop techniques capable of mapping its complex spatiotemporal dynamics in awake, behaving animals. Notably, analytical methods to detect serotonin in situ, including tomography, are widely used but still recognized as limited in terms of their spatiotemporal resolution, their methodological caveats, and their technical limitations when cross-referenced with behavioral studies. To overcome such limitations, genetically encoded serotonin indicators were developed, leading to the introduction of novel imaging modalities that enable researchers to achieve remarkable spatiotemporal resolution in the study of serotonergic circuits in preclinical models of neuropsychiatric disorders. These novel approaches, while remarkably powerful, are also not without limitations. Here, we review the current techniques for detecting and quantifying serotonin in vivo within the brain and discuss how novel approaches such as genetically encoded serotonin indicators will lead to new insights into the roles of serotonergic circuits in health and disease.

KEYWORDS
functional magnetic resonance imaging, genetically encoded fluorescent sensor, microdialysis, positron emission tomography, serotonin, voltammetry

Abbreviations: 3D, three-dimensional; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; AADC, aromatic amino acid decarboxylase; ATD, acute tryptophan depletion; Aβ, amyloid-β; BBB, blood–brain barrier; BM3, flavocytochrome P450-BM3; BOLD, blood oxygen level-dependent; cAMP, cyclic AMP; CE, capillary electrophoresis; CNiFERs, cell-based neurotransmitter fluorescent-engineered reporters; cpGFP, circularly permuted GFP; DAG, diacylglycerol; DMT, N,N-dimethyltryptamine; DPM, dorsal paired medial; DRN, dorsal raphe nucleus; ECD, electrochemical detection; FFN246, a fluorescent false neurotransmitter; fMRI, functional magnetic resonance imaging; FPs, fluorescent proteins; FRET, Förster resonance energy transfer; FSCV, fast-scan cyclic voltammetry; GESIs, genetically encoded serotonin indicators; GPCRs, G protein-coupled receptors; GRIN, gradient index; HPLC, high-performance liquid chromatography; IP3, inositol triphosphate; KCs, Kenyon cells; LSD, d-lysergic acid diethylamide; MAO, monoamine oxidase; MB, mushroom body; mPFC, medial prefrontal cortex; PBPs, bacterial periplasmic binding proteins; PET, positron emission tomography; SERT, sodium-dependent serotonin transporter; SNr, substantia nigra pars reticulata; SSRIs, selective serotonin reuptake inhibitors; TGMs, transglutaminases; TMs, transmembrane domains; TPH, tryptophan hydroxylase; Trp, L-tryptophan; VMAT2, vesicular monoamine transporter type 2.

1 | INTRODUCTION

Serotonin, also known as 5-hydroxytryptamine (5-HT), is an evolutionarily ancient biogenic molecule in the biological world. This biomolecule is widespread among taxonomic kingdoms, as it is found in bacteria, fungi, protozoa, plants, and animals (Bowden et al., 1954; Erspamer & Asero, 1952; Hsu et al., 1986; McGowan et al., 1983; Stijve, 1992). Along with its wide distribution in nature, serotonin also has broad physiological functions ranging from trophic factor and hormone to neurotransmitter (Azmitia, 2020; Erland et al., 2018; Martin et al., 2017; Mawe & Hoffman, 2013; Nichols & Nichols, 2008). In the brain, serotonin acts through receptors that are characterized by diverse downstream signaling pathways modulating important neurological processes, including cognition, learning, memory, mood, sleep, aggression, anxiety, and appetite (Berger et al., 2009; Jacobs & Azmitia, 1992; Mawe & Hoffman, 2013; Nichols & Nichols, 2008). Our understanding of serotonin neurological function has been developed mainly by advancements in methods and techniques for the measurement and visualization of serotonin in the brains of behaving animals. In this review, we first provide historical perspectives on the discovery of serotonin and early techniques used for its detection in the time preceding the development of the first methods for in vivo measurements. We briefly review serotonin metabolism in mammals and discuss major challenges of serotonin measurement in vivo. Then, we review state-of-the-art techniques for serotonin detection and measurement in vivo, including microdialysis, voltammetry, tomography, and genetically encoded fluorescent biosensors. We also discuss major challenges and limitations of the currently available methods and techniques in terms of their spatiotemporal resolution, selectivity, sensitivity, invasiveness, and accessibility. We conclude the review by providing our perspective on emerging tools and potential future directions in serotonin research.

1.1 | History of the discovery of serotonin and techniques for its detection

It has been over 100 years since serotonin attracted the attention of scientists and more than half a century since the development of the first method for in situ visualization of serotonin in the brain (Figure 1). Many methods and techniques that played crucial roles in identifying serotonin and determining its biological function and metabolism in its early days are no longer in use. However, we believe that a brief overview of the early history of serotonin research can help us put the currently used methods into perspective and evaluate the technological progress achieved in the field. This section will review the discovery of serotonin and the history of the pioneering techniques used for serotonin measurements in neuroscience.

Although serotonin is presently known best as a neurotransmitter, it was first identified in blood serum (cell-free fluid from clotted blood) as a vasoconstrictor (Whitaker-Azmitia, 1999). Ludwig and Schmidt were reportedly among the first scientists to observe the physiological effect of serotonin under experimental conditions, reporting their findings as early as 1868 (Göthert, 2013; Page, 1954, 1976; Starke, 1998). These investigators noted that defibrinogenated blood, when infused arterially, increased vascular resistance in perfused dog muscle (Ludwig & Schmidt, 1868). In the following decades, these observations were reproduced and further extended by other scientists, including Stevens and Lee as well as Brodie, who also found that treating plasma with magnesium sulfate, sodium sulfate or sodium citrate prevented its vasoconstrictive effect (Brodie, 1900, 1903; Stevens & Lee, 1884). However, it was initially suggested that the vasoactive ingredient in blood was adrenaline (Trendelenburg, 1910). It was not until 1911–1912 that O'Connor, working on measuring the adrenaline concentration in blood, determined that the unknown vasoconstrictor was distinct from adrenaline and that its release into plasma occurred during blood coagulation, presumably from the platelets (O'Connor, 1912). These results were confirmed in 1913 by two independent studies performed by Janeway and Park and by Stewart and Zucker, using ox carotid arterial strips in a bioassay to detect vasoconstriction (Janeway & Park, 1912; Stewart & Zucker, 1913). However, these early studies did not aim to isolate or identify the active ingredient. More systematic research began in 1933 when Vialli and Erspamer reported the discovery of enterochromaffin cells in the gastrointestinal tracts of a variety of vertebrate species (Vialli & Erspamer, 1933). This discovery resulted in the extraction of a novel substance from enterochromaffin cells of the rabbit gut; this substance, named enteramine, was demonstrated to induce smooth muscle contraction of the rat and mouse gut and the rat uterus (Vialli & Erspamer, 1937). In 1946, Erspamer suggested the indolic nature of enteramine based on comparative analysis using indole alkaloids as references, although he was not able to determine its exact chemical structure (Erspamer, 1946). Independent of Erspamer, Page was working on the isolation of an interfering vasoconstrictor associated with blood clotting to study the pathogenesis of essential hypertension. In 1948, as a result of this effort, Rapport, Green, and Page isolated a compound from bovine serum, and because of its discovered function as a vasoconstrictor, they called it serotonin (Page et al., 1948; Rapport et al., 1948). The word serotonin was coined by Corcoran, a colleague of Page, and was formed by combining the Latin word "serum," referring to the substance in which it was found, with the Greek word "tonic," referring to its earliest identified function. In 1949, Rapport identified crystallized serotonin as a creatinine sulfate complex and suggested its chemical identity to be 5-HT (Rapport et al., 1948). Subsequent chemical synthesis of serotonin by Speeter in 1950 enabled large-scale production of this substance by the Upjohn Company and Abbott, making it available for numerous pharmacological and biological studies in various systems (Hamlin & Fischer, 1951; Rapport, 1997; Speeter et al., 1951). Eventually, the isolation of enteramine in a pure form allowed Erspamer and Asero to identify it as 5-HT (Erspamer & Asero, 1952). It was suggested by Bacq that the terms "enteramine" and "serotonin" should be replaced with 5-HT, as the original names are not accurate in terms of the specificity of location and/or action. However, the name serotonin has been retained, and in the modern

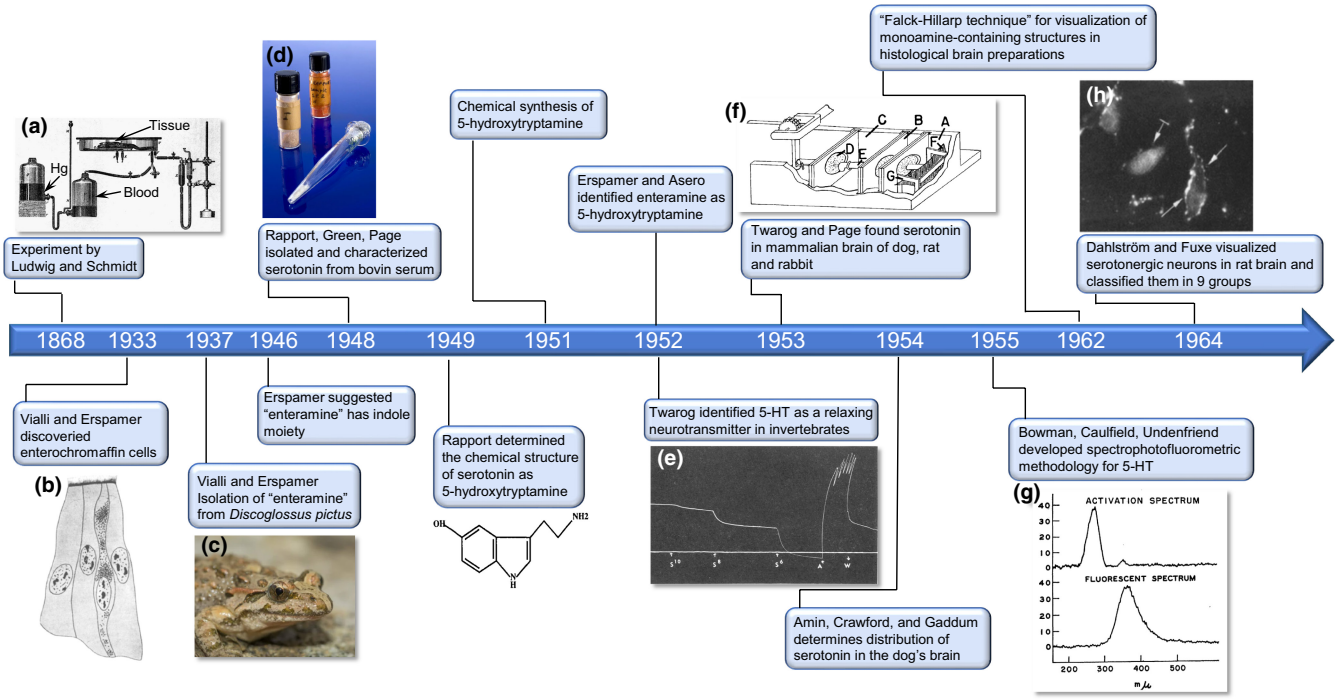

FIGURE 1 Brief history of serotonin discovery and techniques for serotonin detection. (a) Wood engraving of the apparatus for arterial infusion of isolated living mammalian muscle used by Ludwig and Schmidt (adapted from Boettcher et al., 2003). (b) Enterochromaffin cell drawings of a common frog (*Rana esculenta*; adapted from ref https://samorini.it/novita-sul-sito/the-serotonin-of-vittorio-erspamer/). (c) Image of *Discoglossus pictus* (from https://www.shutterstock.com). (d) Images of serotonin creatinine sulfate stored in the Science Museum in London (presumably one of these vials contains the first ever prepared crystal of serotonin creatinine sulfate saved by Maurice Rapport during the earliest attempts at serotonin purification from bovine serum in 1948. Maurice Rapport sent 0.4 mg of serotonin crystals to Dr. Henry McIlwain for the Science Museum in London in 1985). (e) Contractile response of the *Mytilus* muscle upon administration of serotonin and acetylcholine (the original manuscript was submitted in 1952 but not published until 1954, Twarog, 1988; adapted from Twarog, 1954). (f) Schematic of the muscle chamber used by B. Twarog for recording the contractile response (adapted from Twarog, 1954). (g) Fluorescence spectrum of 5-hydroxytryptamine (5-HT) recorded using the first spectrophotofluorometer (adapted from Bowman et al., 1955). (h) A 5-HT nerve cell (with weak yellow fluorescence) is closely surrounded by fine, catecholamine terminals (↗), while another 5-HT cell body shows no close contact with such fibers (↥) (adapted from Fuxe, 1965).

literature (including this review), the terms "serotonin" and "5-HT" are used interchangeably.

The methods for selective identification of active ingredients in tissue extracts played a crucial role in establishing serotonin as an important neurotransmitter, as well as for understanding its roles, metabolism, and distribution within the brain. The oldest bioassays for serotonin were based on isolated tissues from mammals and mollusks. By the early 1950s, a number of sensitive and quantitative bioassays were developed and used by Erspamer, Page, Welsh, and Guddam to determine the serotonin concentrations in a multitude of preparations. In 1952 (although the original manuscript reporting this finding was not published until 1954), Twarog, while working at Harvard University, identified 5-HT as a relaxing neurotransmitter in *Mytilus* (Twarog, 1954), making it the third neurotransmitter discovered after acetylcholine (1933) and norepinephrine (1946), whose presence was confirmed at the peripheral level (López-Muñoz & Alamo, 2009). This discovery allowed Twarog to suggest that 5-HT might be present in the brains of mammals. Correspondingly, in 1953, Twarog and Page found serotonin in the brains of several mammals, specifically dogs, rats, and rabbits (Twarog & Page, 1953), by using an ultrasensitive bioassay based on the isolated heart of *Venus mercenaria* (now renamed *Mercenaria mercenaria*). Independently, in 1954, Amin, Crawford, and Gaddum used the isolated estrous uterus in a bioassay to determine the distribution of 5-HT in the brain of the dog (Amin et al., 1954). These seminal discoveries brought 5-HT into the field of neuroscience and triggered a wealth of studies focused on its function in the brain.

The emergence of novel instrumental and analytical methods for the detection of low-molecular-weight compounds, which began to flourish in the late 1950s, significantly expanded the toolkit for studying serotonin function in the brain and eventually replaced tissue-based bioassays. One of the first milestones in the technological advancement of analytical methods for serotonin detection was the development of the spectrophotofluorometer in 1955. This new optical hardware enabled sensitive methods for measuring the concentration of monoamines, including serotonin, in the brain, offering higher throughput and greater simplicity than isolated-tissue bioassays (Bowman et al., 1955; Crawford & Rudd, 1962). As an indole, serotonin can be detected directly because of its native ultraviolet fluorescence (with excitation and emission maxima at 297

and 337 nm, respectively), which is typical of aromatic compounds (Chattopadhyay et al., 1996). However, the reaction of serotonin with other chemicals, such as ninhydrin and o-phthalaldehyde, was used to increase the selectivity and sensitivity of direct measurements by an order of magnitude (Maickel et al., 1968; Snyder et al., 1965). Nevertheless, the presence of interfering or cross-reactive compounds in biological samples limits the application of spectrophotofluorometric methods. Further development of more advanced techniques for molecular separation and detection, including chromatography, mass spectrometry, immunoassays, etc. (Peskar & Spector, 1973; Saavedra et al., 1973) made it possible not only to overcome these limitations but also to improve the specificity and sensitivity of serotonin detection. Analytical methods are still commonly used for serotonin measurements in various biological samples and have provided a wealth of knowledge about serotonin physiology in health and disease (Sugiyama et al., 2019). However, these methods often rely on the extraction of serotonin from tissue homogenates and therefore are not compatible with in vivo measurements. The first method that enabled in situ visualization of monoamines, including serotonin, with subcellular resolution was the Falck-Hillarp technique, introduced in 1962 (Falck et al., 1982; Figure 1). The visualization of catecholamines and serotonin in situ was achieved because of the formation of yellow–green fluorescent isoquinoline molecules during the exposure of freeze-dried tissue to formaldehyde vapor. Utilizing this technique in 1964, Dahlström and Fuxe visualized and mapped serotonergic neurons in the rat brain, classifying them into nine distinct clusters designated B1 through B9 (Dahlström & Fuxe, 1964). However, the Falck-Hillarp technique was not widely adopted because of the need for special equipment and highly skilled personnel to obtain optimal staining results; therefore, it was quickly replaced in the 1980s by immunohistochemistry, promoted by the development of antibodies for 5-HT by Steinbusch et al. (1978). At present, immunohistochemistry remains one of the major methods for in situ imaging of serotonin, along with more recently developed imaging mass spectrometry (Sugiyama et al., 2020).

The early methods for serotonin detection in the brain as reviewed in this section enabled important studies that contributed to serotonin discovery and elucidating its distribution and occurrence in the brain; however, they were not compatible with in vivo measurements. Electrochemical and microdialysis techniques reported in the early 1970s (Delgado et al., 1972; Kissinger et al., 1973; McCreery et al., 1974) were the first methods used to detect and measure serotonin in vivo in behaving animals (Marsden et al., 1979; Ungerstedt & Hallström, 1987). At present, these remain popular approaches for basic neuroscience research (Figure 2). The very first documented measurements of serotonin distribution and metabolism in the human brain were achieved using positron emission tomography (PET; Wagner, 1986) and functional magnetic resonance imaging (fMRI; Krämer et al., 2011; Wingen et al., 2008), which are the major techniques for biomedical and clinical investigations of the serotonergic system. The most recently introduced technology for visualizing serotonin dynamics in vivo relies on newly developed genetically encoded fluorescent biosensors. Fluorescent biosensors are the most rapidly evolving tools, with great potential for multiplex imaging in behaving animals. In the following sections, we will review each category of state-of-the-art technologies in detail, discussing their major advantages and drawbacks.

1.2 | Metabolism and physiological functions of serotonin

The major source of serotonin in animals is L-tryptophan (Trp), which is supplied through food as an essential amino acid. The free form of Trp is preferentially transported across the blood–brain barrier (BBB) by a large neutral amino acid carrier protein

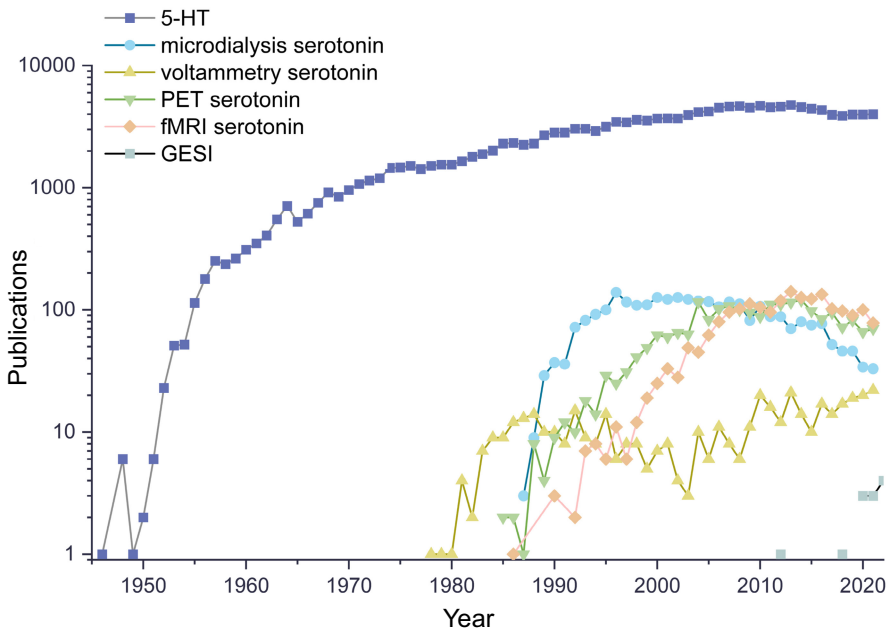

FIGURE 2 Trends in the development and application of technologies for serotonin detection and imaging in vivo. The number of scientific articles per corresponding year was identified by a PubMed search using the following keywords: serotonin (5-HT); microdialysis + serotonin (5-HT); voltammetry + serotonin (5-HT); PET + serotonin (5-HT); fMRI + serotonin (5-HT) (serotonin and 5-HT keywords produce almost identical results). An online search for "Genetically Encoded Serotonin Indicators" (abbreviated here as GESI) was performed using various search engines and verified manually (including non-peer-reviewed papers from biorxiv.org). 5-HT, 5-hydroxytryptamine.

(Höglund et al., 2019). Mediated by high-affinity transporters (Gyawali & Kang, 2021), such as the SLC7A5 transporter in humans (Scalise et al., 2018), tryptophan is transported into cells (Ruddick et al., 2006). The biosynthesis of serotonin occurs in the terminals of serotonergic neurons and starts with the conversion of Trp to 5-hydroxytryptophan (5-HTP) catalyzed by the rate-limiting enzyme tryptophan hydroxylase (TPH; Figure 3a,b). TPH has two representative forms: the broadly expressed protein Tph1 and the central nervous system-enriched protein Tph2 (Côté et al., 2003). The subsequent metabolic step in serotonin synthesis involves the decarboxylation of 5-HTP by aromatic amino acid decarboxylase (AADC) in the cytosol. Approximately 1%–2% of Trp taken up in healthy individuals is converted into serotonin in the serotonin pathway (Comai et al., 2019). Cytosolic serotonin is packed into presynaptic vesicles by membrane-embedded vesicular monoamine transporter type 2 (VMAT2; Eiden & Weihe, 2011). After release into the synaptic cleft, serotonin binds to serotonin receptors on post-synaptic neurons or to presynaptic autoreceptors. Extracellular serotonin levels are under precise control by multiple regulatory systems, which are crucial for proper brain function. Serotonin in the extracellular space is mainly cleared out by the sodium-dependent *serotonin* transporter (SERT), which is expressed in presynaptic neurons and astrocytes (Kanner & Zomot, 2008; Schloss & Williams, 1998; Figure 3b). Although SERT expression in astrocytes has been reported by several groups (Inazu et al., 2001; Kubota et al., 2001; Malynn et al., 2013), these findings remain limited and require further validation. In addition to SERT, it has been shown that organic cation transporters (Baganz et al., 2008; Schmitt et al., 2003), plasma membrane monoamine transporters (Vieira & Wang, 2021; Zhou et al., 2007), dopamine transporters (Daws, 2009; Zhou et al., 2002), and norepinephrine transporters (Daws, 2009; Daws et al., 1998) are also involved in the serotonin clearance process from extracellular space. These "non-traditional" serotonin-clearing proteins play an important role when extracellular serotonin levels are excessive, especially as a complementary mechanism when SERT function is deficient (Baganz et al., 2008; Daws, 2009; Pan et al., 2001). The metabolism of serotonin is completed by its oxidation into 5-hydroxyindoleacetic acid (5-HIAA), carried out by monoamine oxidase (MAO) localized in the outer mitochondrial membrane (Figure 3a,b). 5-HIAA does not have any biological activity and is eliminated from organisms by the kidney (Shih et al., 1999; Singh et al., 1999). Serotonin synthesis, release, and reuptake in a single serotonergic neuron terminal can be described by a mathematical model proposed by Best et al. (Best et al., 2010).

In mammals, approximately 90%–95% of all serotonin in the body is synthesized and secreted by enterochromaffin cells within the gastrointestinal mucosa, and only ~5% of serotonin is found in the central nervous system (Banskota et al., 2019; Kim & Camilleri, 2000). Since serotonin cannot cross the BBB, the functions of the central serotonergic system and the peripheral serotonergic system are separate. In the peripheral serotonergic system, although this substance was initially thought to work as a vasoconstrictor, current research on serotonin continually brings new biological insight into its diverse physiological functions (Lv & Liu, 2017). Serotonin appears to contribute significantly to the function of the gut, for example, by stimulating gastrointestinal motility events such as propulsion and segmentation (Martin et al., 2017; Spohn & Mawe, 2017). In addition to the gut, serotonin is also involved in the regulation of glucose homeostasis, lipid metabolism, bone density, platelet aggregation, and coronary vasoconstriction (Martin et al., 2017; Mawe & Hoffman, 2013; Spohn & Mawe, 2017).

In addition to working as a signaling molecule in the peripheral serotonergic system, serotonin is perhaps best known as a neurotransmitter in the brains of all species studied to date. In mammals, it was found to be involved in mood regulation (Lesch et al., 1996), sleep/wake homeostatic regulation (Portas et al., 1998), reward processing (Li et al., 2016; Liu et al., 2014), pain (Bardin, 2011), and memory (Fernandez et al., 2016). During the sleep/wake cycle, the concentrations of 5-HT in the dorsal raphe nucleus (DRN) are upregulated in waking and down-regulated during rapid eye movement sleep and slow-wave sleep in mice (Portas et al., 1998). In the anticipatory and consummatory phases of reward responses, serotonergic neurons in the DRN positively encode a wide range of reward signals in behaving mice (Li et al., 2016). In addition, the dysregulation of serotonin levels in the nervous system is related to mental disorders such as depression (Meltzer, 1990; Millan, 2005; Owens & Nemeroff, 1994) and anxiety (Charney et al., 1987; Eison, 1990; Lesch et al., 1996). In some cases, dysregulation of serotonin metabolism can be associated with polymorphisms in the SERT and TPH2 genes, resulting in unipolar depression, bipolar disorder, anxiety, and suicidality (Caspi et al., 2003; Heils et al., 1996; Lesch et al., 1996; Lin et al., 2007; Zhang et al., 2005). Therefore, targeting central serotonergic activity is one of the generally accepted strategies for treating psychiatric disorders. For example, there are two major types of antidepressant drugs aimed at proteins involved in serotonergic activity: selective serotonin reuptake inhibitors (SSRIs; Millan, 2005; Vaswani et al., 2003) and MAO inhibitors (Shopsin et al., 1976; Thase et al., 1992). SSRIs, the most common class of antidepressants, selectively increase extracellular serotonin levels by inhibiting SERT (Fuller, 1994). SERT is mainly responsible for determining the duration and strength of the 5-HT signal by dynamically removing 5-HT from the extracellular space (Figure 3b). Because of this function, many treatments for serotonin-related disorders primarily target SERT; for example, this is true of SSRIs. The other type of antidepressant targets MAO, one of the major enzymes involved in the degradation of free serotonin. Two types of MAO exist in mammals, namely, MAO-A and MAO-B (Green, 2008; Shih et al., 1999). MAO-A has a higher affinity for serotonin than MAO-B (Shih et al., 1999; Shih & Thompson, 1999). MAO-A and MAO-B differ in the tissue types where they are expressed, the developmental stages when they are expressed, and their related functions (Edmondson et al., 2004; Shih & Thompson, 1999). Both isoenzymes deaminate monoamine neurotransmitters such as serotonin, dopamine, and norepinephrine that are not stored in vesicles (Youdim et al., 2006). Oxidation by MAO breaks down serotonin to 5-HIAA, thus decreasing the

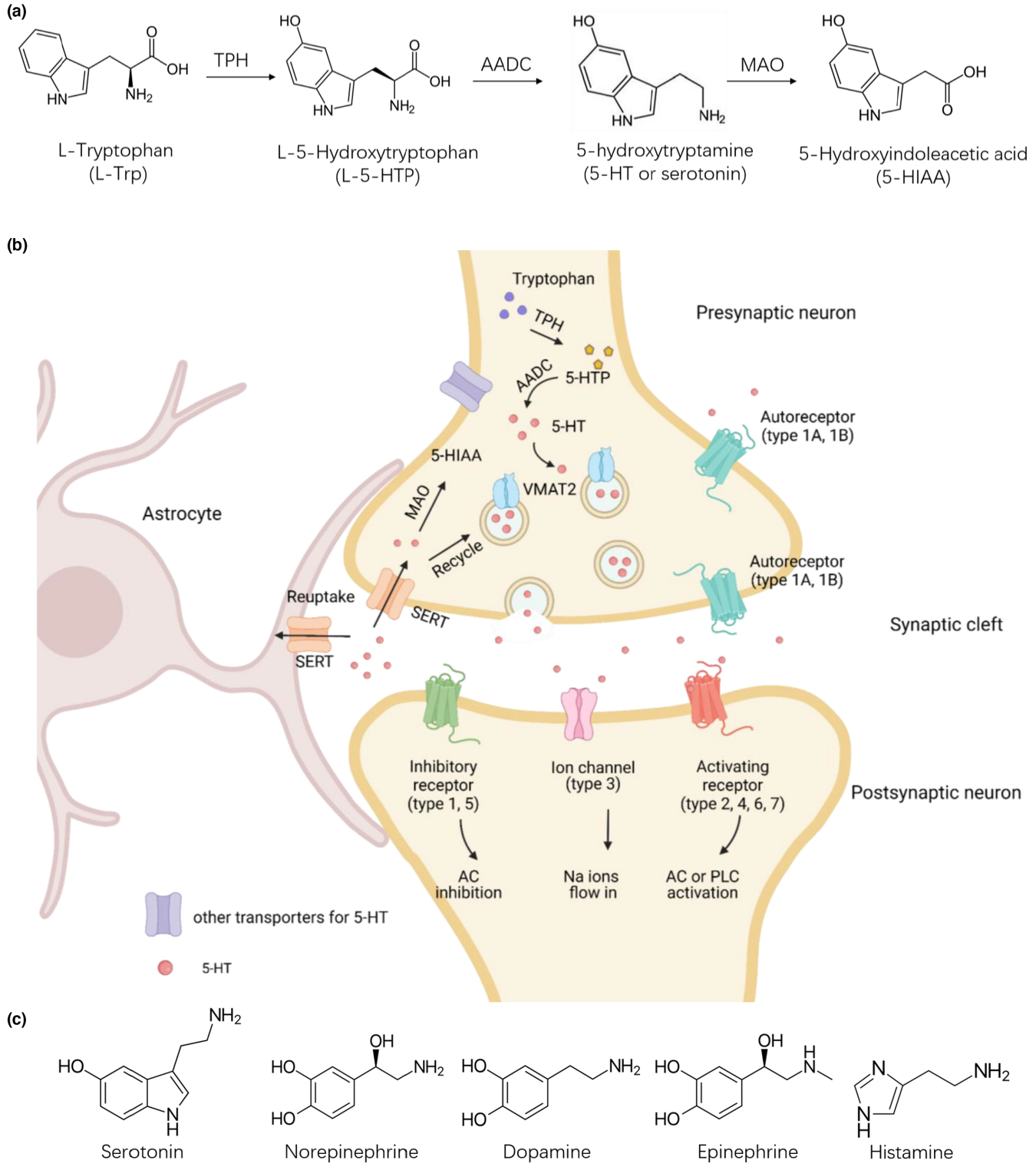

FIGURE 3 Serotonin metabolism in the nervous system. (a) Chemical reactions illustrating serotonin biosynthesis and degradation. (b) Schematic representation of serotonin metabolism showing key serotonin binding proteins and transporters. The expression of sodium-dependent serotonin transporter (SERT) in astrocytes has been reported (Inazu et al., 2001; Kubota et al., 2001; Malynn et al., 2013) but remains to be further clarified. AADC, aromatic amino acid decarboxylase; MAO, monoamine oxidase; TPH, tryptophan hydroxylase. (c) Serotonin and monoamine neurotransmitters share similar chemical structures.

availability of serotonin in neurons (Youdim et al., 2006; Figure 3a,b). Inhibition of MAO increases serotonin availability in neurons, and therefore, MAO inhibitors are applied as antidepressants in clinical treatment (Youdim et al., 2006). The other prospective drug targets for various neuropsychiatric diseases are 5-HT receptors, with a particular focus on 5-HT_{2A} receptors, which are targeted by many

psychedelic drugs, including psilocybin, mescaline, LSD (d-lysergic acid diethylamide), and DMT (N,N-dimethyltryptamine), among others (Ballentine et al., 2022; McClure-Begley & Roth, 2022). For example, psilocybin, a naturally occurring psychedelic prodrug, was granted FDA breakthrough therapy designation and is currently in phase II clinical trials for the treatment of anxiety and depression (Carhart-Harris et al., 2021; Griffiths et al., 2016; Ross et al., 2016). Recent success in using psychedelics as antidepressant drugs creates a need for a more comprehensive understanding of the neurobiological mechanism behind their action and thus promotes further development of novel methods for serotonin detection.

In addition to its role as a neurotransmitter, serotonin is also utilized as a substrate for post-translational modification by covalent linkage to glutamine residues of proteins through the enzymatic actions of transglutaminases (TGMs). This process, known as serotonylation, can influence the half-life of proteins such as small GTPases, including members of the Rho and Rab families of proteins (Muma & Kapadia, 2020). The molecular roles and cellular impact of serotonylation are beyond the scope of this essay but are the focus of several reviews (Bader, 2019; Muma & Mi, 2015).

1.3 | Challenges in detecting serotonin

Despite the critical importance of 5-HT, it remains one of the most mysterious neurotransmitters because of our poorly developed understanding of 5-HT spatiotemporal dynamics in the nervous system in the context of behavior. There are five challenges that complicate the process of assessing the function of serotonin in the central serotonergic system: (i) extracellular serotonin concentration is relatively low compared to most other neurotransmitters; (ii) serotonin transmission occurs at drastically different time scales; (iii) different types of serotonin receptors have different downstream functions; (iv) serotonin shares high chemical structure similarity with other monoamine neurotransmitters; and (v) location of serotonergic neurons in the brain.

The comprehensive understanding of serotonin function in vivo requires methods and techniques that enable highly selective measurements of serotonin transients with high sensitivity and spatiotemporal resolution, which represent a great challenge for most well-established techniques for serotonin detection. Investigation of serotonin homeostasis is not only crucial for fundamental neuroscience but also necessary for the development of novel and more effective therapies for depression and anxiety. Since the five major challenges listed above represent the core problem of serotonin detection in vivo, we will briefly review their biological aspects.

First, the extracellular level of serotonin is relatively low compared to those of most other neurotransmitters. For instance, the basal brain serotonin concentration of rats measured using microdialysis or voltammetry techniques varies on the nanomolar order of magnitude, from 0.180 nM in the medial prefrontal cortex (mPFC) to 5 nM in the frontal cortex, 10 nM in the DRN, and 68.4 nM in the striatum (Table 1; Crespi et al., 1988; Klomp et al., 2014; Xu et al., 2006). For comparison, in some brain regions, such as the nucleus accumbens and caudate–putamen, the dopamine concentration is more than 10-fold higher than the concentration of serotonin in wild-type mice (Shen et al., 2004).

Several factors account for the basal extracellular serotonin level in the brain. The foremost contributing factor is the strict regulation of the nervous system. The simultaneous role of serotonin in many regulatory systems within the nervous system determines that the 5-HT concentration needs to be strictly regulated to maintain homeostasis (Best et al., 2010). This strict regulation leads to a relatively low extracellular concentration of serotonin in most brain regions compared with other commonly studied neurotransmitters, such as dopamine, GABA, and glutamate (Zhu et al., 2011). Many proteins are involved in this regulatory process, such as serotonin receptors, SERT, MAO, and VMAT2 (Figure 3b). Following its reuptake by SERT, serotonin is delivered into vesicles for storage or enzymatically degraded by MAO. VMAT2, an integral membrane protein, transports monoamine neurotransmitters such as serotonin, dopamine, norepinephrine, and histamine from the cellular cytosol into synaptic

TABLE 1 Serotonin concentrations in different brain regions.

Method	Brain region	Concentration	Reference
Voltammetry (Nafion-coated carbon fiber electrode)	Frontal cortex (rat)	5 nM	Crespi et al. (1988)
Voltammetry (Nafion-coated carbon fiber electrode)	Dorsal raphe (rat)	10 nM	Crespi et al. (1988)
Microdialysis (combined with HPLC-ECD)	CPu (mouse)	0.182 nM	Shen et al. (2004)
Microdialysis (combined with HPLC-ECD)	PFc (mouse)	0.305 nM	Shen et al. (2004)
Microdialysis (combined with HPLC-ECD)	NAc (mouse)	0.119 nM	Shen et al. (2004)
Microdialysis (combined with HPLC-ECD)	Striatum (rat)	68.4 ± 3.2 nM	Xu et al. (2006)
Microdialysis (combined with HPLC-ECD)	mPFC (rat)	0.170 nM	Klomp et al. (2014)
FSCAV (Nafion-coated carbon fiber microelectrodes)	CA2 of HC (mouse)	64.9 ± 2.3 nM	Abdalla et al. (2017)
FSCV/FSCAV (Nafion-modified carbon fiber electrode)	HC (mouse)	62.5 ± 1.8 nM	Saylor et al. (2019)

Abbreviations: CPu, caudate–putamen; ECD, electrochemical detection; FSCAV, fast-scan controlled-adsorption voltammetry; FSCV, fast-scan cyclic voltammetry; HC, hippocampus; HPLC, high-performance liquid chromatography; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; PFc, prefrontal cortex.

vesicles (Guillot & Miller, 2009). The tight regulatory mechanism of 5-HT release and clearance results in a relatively low extracellular 5-HT concentration compared to most of other neurotransmitters. Another reason for this low extracellular level of serotonin is that the total number of serotonergic neurons is low, totaling 300000–450000 serotonergic neurons in human brains (Baker, Halliday, Halasz, et al., 1991; Baker, Halliday, Hornung, et al., 1991; Halliday et al., 1988; Hornung, 2003) and 20000–26000 in rat and mouse brains (Descarries et al., 1982; Ishimura et al., 1988; Jacobs & Azmitia, 1992). Such low number of serotonergic neurons limits the synthesis and release of serotonin. Thus, to monitor fluctuations in 5-HT levels and investigate the serotonergic system, detection methods must be sufficiently sensitive to detect the physiological concentration of serotonin in the nanomolar range.

The second aspect concerns the two different spatiotemporal scales of serotonin chemical signaling: volume transmission and synaptic transmission. Volume transmission is a widespread, diffuse mode of intercellular communication occurring on extended time scales (Bunin & Wightman, 1998; Jennings, 2013). For this type of transmission, serotonergic neurons project widely to forebrain structures such as the amygdala, the hippocampus, and all regions of the neocortex and striatum (Hornung, 2003). This signaling pathway with release followed by diffusion resulting in widespread receptor activation on targeted cells is called volume transmission or paracrine signaling (Hensler, 2006; Hornung, 2003). For this slow, diffusion-based transmission, assessing large volumes of brain tissue will increase the chance of cross-talk with other chemically similar neurotransmitters; thus, there are higher requirements for the specificity of detection. Unlike volume transmission, chemical synaptic transmission usually occurs on the millisecond timescale and is influenced by environmental and intrinsic factors. For this type of transmission, information from the presynaptic neuron is transmitted to the dendrites or cell body of the post-synaptic neuron by secreting serotonin within the synaptic cleft. The width of the synaptic cleft is approximately 20 nm (Ahmari & Smith, 2002; Lučić et al., 2005; Schikorski & Stevens, 1997). The nature of synaptic transmission, with a fast timescale and narrow physical space, offers the unique challenge of detecting serotonin release activity in a single synapse. As such, detection methods with extraordinary temporal resolution and spatial resolution are required to investigate the dynamics of serotonin synaptic transmission underlying neural activity. Currently, methods to detect serotonin activity in awake, behaving animals with single synapse resolution are lacking.

Third, the task of developing a comprehensive understanding of serotonin signaling is complicated by the identification of numerous serotonin receptor family members, many with complex functions. There are seven distinct classes of serotonin receptors, including 14 subtypes: all subtypes are G protein-coupled receptors (GPCRs) except for 5-HT_3, which is a ligand-gated cation channel. Serotonin GPCRs couple to three signaling pathways through Gαq, Gαs, and Gαi/o proteins. The 5-HT_2 family of receptors is coupled with Gαq, and its activation results in the formation of second messengers such as diacylglycerol (DAG) and inositol triphosphate (IP3), which act as signaling molecules to activate protein kinase C and elevate intracellular calcium (Gresch & Kitainda, 2021; Nichols & Nichols, 2008). Types 4, 6, and 7 of 5-HT receptors are Gαs protein-coupled, and activation leads to stimulation of adenylyl cyclases. Adenylyl cyclase converts ATP to cyclic AMP (cAMP), which is a ubiquitous second messenger that interacts with a considerable number of targets (including cyclic nucleotide-gated ion channels and protein kinase A; Gresch & Kitainda, 2021; Nichols & Nichols, 2008). Gαi/o-coupled receptors, encompassing 5-HT_1 and 5-HT_5 receptors, are defined by inhibiting adenylyl cyclase and decreasing the production of cAMP (Gresch & Kitainda, 2021; Nichols & Nichols, 2008). In particular, as a ligand-gated cation channel, the activation of the 5-HT_3 receptor by serotonin results in an opening of the ion pore and rapid activation, followed by desensitization of the inward current (Gresch & Kitainda, 2021; Nichols & Nichols, 2008). Evidently, binding of serotonin to its receptors results in activation of distinct intracellular signal transduction pathways and, in turn, drives diverse physiological consequences. Notably, such diverse physiological consequences of serotonergic signaling are highly relevant to the actions of psychotropic drugs. Currently, most detection methods merely reflect fluctuations in the level of serotonin, not the biological meanings behind them. Furthermore, complex pharmacological interactions, including inverse agonism and functional selectivity (also known as biased agonism; Berg & Clarke, 2018), can further expand on the causal, physiological impact of serotonin signaling across a molecular landscape with a multitude of receptor subtypes, beyond merely measuring the presence of serotonin alone within cells and tissues. The multiple physiological effects of serotonin receptors increase the difficulty of analyzing the role of serotonin level changes involved in certain behaviors, which represents a requirement for detection with the resolution of the receptor subtype level.

Fourth, as a monoamine, serotonin has a similar chemical structure to other monoamine neurotransmitters, such as dopamine, norepinephrine, histamine, and epinephrine (Figure 3c). This similarity increases the chance for cross-talk when detecting serotonin in the extracellular space with other monoamine neurotransmitters. For instance, detection methods based on electrochemical reactions such as fast-scan cyclic voltammetry (FSCV) suffer from this limitation. Distinguishing serotonin and dopamine during the detection process is one of the biggest challenges for FSCV (Robke et al., 2020). Furthermore, long-term measurement of serotonin using voltammetric techniques is limited by electrode fouling, a process in which the highly reactive products of serotonin oxidation at detection electrodes impair measurement accuracy and stability (Hashemi et al., 2009; Puthongkham & Venton, 2020). Thus, the chemical properties of serotonin require highly specific detection methods.

Finally, the serotonergic system in the central nervous system of mammals mainly originates in the raphe nuclei, a moderate-sized cluster of nuclei found in the brainstem (Hornung, 2003; Steinbusch, 1981). Among the raphe nuclei, the DRN, a minute hub in the center of the medulla, contains the most serotonin-producing neurons, which send ascending projections into multiple brain regions (Liu et al., 2014). The area around the DRN was reported to have

the highest concentration of 5-HT in the brain (Ishimura et al., 1988; Sugiyama et al., 2019). The level of serotonin in the whole brain is largely determined by secretion from serotonergic neurons in the DRN, and therefore, researchers have largely targeted their studies to this region of the brain to understand serotonergic signaling in the brain (El Mansari et al., 2015; Li et al., 2016; Liu et al., 2014; McDevitt et al., 2014; Petrucci et al., 2021). However, the DRN is located underneath a dense network of core vessels and passages, such as the sagittal sinus and the cerebral aqueduct. This property complicates the experimental measurement of extracellular serotonin levels in the DRN, especially for methods that require implantation of instruments into the brain, which means the risk of substantial damage to the neighboring vasculature and the cerebral aqueduct.

Resolving all these questions requires that detection methods provide high spatiotemporal resolution and high sensitivity while being non-invasive. In addition, the ability to take recurring measurements and observe long-term properties must be taken into account. The purpose of measuring 5-HT is to deeply understand serotonin dynamics in vivo and correlate it with behaviors, especially in chronic disease models. Behavioral studies require tracing serotonin levels for a long time, which also demands methods capable of recurrent and continuous measurement. During past decades, in an effort to overcome these challenges, many measuring techniques have been developed and advanced to detect serotonin dynamics. Here, we review state-of-the-art methods for detecting serotonin in the central serotonergic system, including traditional analytical methods and fluorescent methods, particularly genetically encoded fluorescent sensors. We also discuss the advantages and disadvantages of each method for serotonin detection.

2 | INSTRUMENT-BASED METHODS FOR SEROTONIN DETECTION

In previous decades, scientists have developed multiple technologies to measure changes in serotonin levels in the brain in vivo (Figure 2). The most notable technique is microdialysis, which was developed in the 1970s and is still of widespread use in neuroscience research (Anderzhanova & Wotjak, 2013; Chefer et al., 2009). Furthermore, electrochemical techniques such as amperometry and cyclic voltammetry have also been employed, both of which detect electric currents induced by serotonin oxidation on the detection surfaces of measuring electrodes. Currently, these remain widely used in neuroscience studies, and their spatial resolution is effective at tissue-level, volumetric transmission based on passive diffusion of 5-HT to the implanted probes. Another type of measuring tool is tomography, represented by fMRI and PET. These techniques are less physically invasive than analytical methods and are commonly used for clinical and biomedical studies in humans. The major advantage of tomography is the ability to perform brain-wide quantitative imaging of neuroreceptor binding and neurotransmitter release. More recently, the development of genetically encoded fluorescent sensors has facilitated the visualization of serotonin in vivo. These sensors provide high spatiotemporal resolution, sensitivity, and specificity. Below, we will discuss all of these techniques and compare them in further detail.

2.1 | Analytical methods: Microdialysis and electrochemistry

2.1.1 | Microdialysis

Microdialysis is a widely used analytical method for concentration measurements of small molecules in the extracellular space of different tissues (Figure 4a; Ungerstedt, 1991). This technique is particularly well established for qualitative and quantitative detection of neurotransmitters (Schultz & Kennedy, 2008), including dopamine (Shen et al., 2004), GABA (Nyitrai et al., 2006), glutamate (Nyitrai et al., 2006), serotonin (Zhang et al., 2013), noradrenaline (Ihalainen et al., 1999), acetylcholine (Damsma et al., 1987), and norepinephrine (Abercrombie et al., 1988). Microdialysis

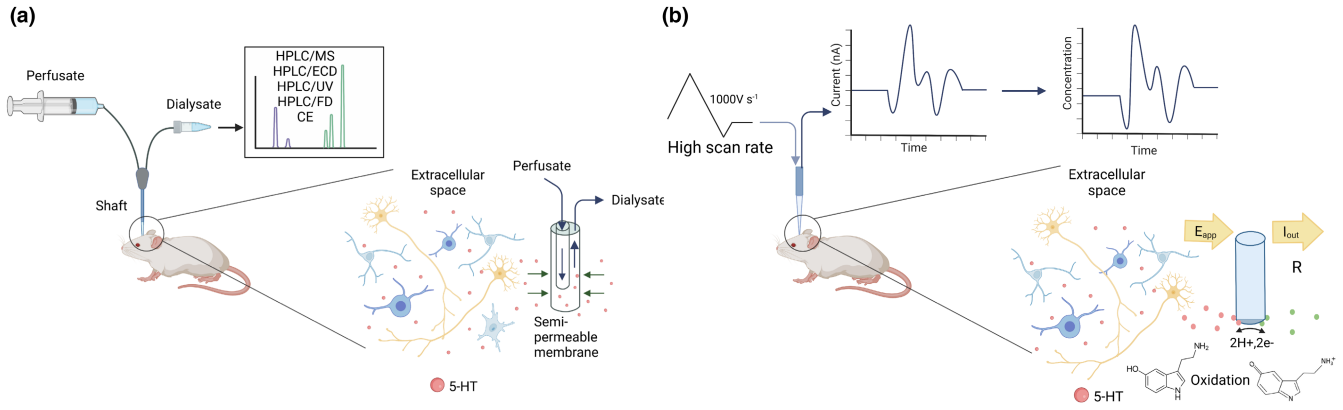

FIGURE 4 Schematic representations of analytical methods for serotonin detection in the brain in vivo. (a) Microdialysis measurements coupled with MS or optical detection. (b) Fast-scan cyclic voltammetry measurements. CE, capillary electrophoresis; ECD, electrochemical detection; FD, fluorescent detection; HPLC, high-performance liquid chromatography; MS, mass spectrometry; UV, ultraviolet absorption detector.

is compatible with in vivo measurements in many neuroscience model organisms, such as mice (Ihalainen et al., 1999), rats (Damsma et al., 1987), rhesus monkeys (Saunders et al., 1994), common marmosets (Nomoto et al., 1994), and even humans (Hillered et al., 1990). Extracellular neurotransmitters are collected using a microdialysis probe containing perfusion liquid for neurotransmitter extraction. The probe can be inserted into organotypic slice cultures, acute brain slices, or freely moving animals. At the tip of the probe (ranging from approximately 200 to 500 μm in diameter, Portas et al., 2000; Sørensen et al., 2013), a semipermeable membrane with a molecular weight cutoff (the maximum permitted value, measuring 13–60 kDa or 100, 1000, or 3000 kDa for large molecules, Jadhav et al., 2016) allows free-floating solutes such as serotonin to conduct across by passive diffusion. Then, the dialysate is collected via a collection device, followed by separation and detection. High-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the most common separation methods used in microdialysis analysis (Anderzhanova & Wotjak, 2013; Lunte, 2000). After separation, detection methods such as electrochemical detection (ECD), fluorescent detection, ultraviolet absorption detector, and mass spectrometry are employed for the analysis of microdialysis samples (Anderzhanova & Wotjak, 2013; Lunte, 2000). Because of efficient analytical techniques for the separation, detection, and quantification of analytes, this method is relatively specific and has a detection threshold of approximately 1 fmol/sample with a signal-to-noise ratio of at least 2 (Shen et al., 2004).

This traditional tool has allowed for elucidation of the essential contribution of serotonin to regulating the normal physiological process, pathophysiology, and treatment of various psychiatric disorders. For example, many microdialysis measurements of serotonin level fluctuations during the sleep/wake cycle have shown that serotonin plays a complex and essential role in sleep facilitation and inhibition (Portas et al., 2000). In addition, microdialysis is a reliable approach for drug pharmacokinetics based on the characteristics of the perfusion system allowing drug delivery along with perfusate. The mechanism of SSRIs has been studied by microdialysis measurements of extracellular serotonin fluctuations induced by administration of various drugs in regions such as the frontal cortex, ventral hippocampus, raphe nuclei, and striatum (Fuller, 1994; Malagié et al., 1995; Perry & Fuller, 1992). For example, a meta-analysis of microdialysis studies of 5-HT concentration during the course of subchronic and chronic SSRI treatment revealed that within the first week of SSRI treatment, extracellular 5-HT concentrations drop in the frontal cortex followed by an up to ~350% increase compared to prior treatment baseline over the next 2 weeks of SSRI administration (Fritze et al., 2017). On the other hand, in the hippocampus, prefrontal cortex, nucleus accumbens, and ventral tegmental area, 5-HT levels were elevated within the first 3 days of SSRI administration (Fritze et al., 2017). However, several other studies utilizing microdialysis have demonstrated a decline in 5-HT levels in the serotonergic terminal areas upon chronic SSRI administration, such as citalopram and fluoxetine, although these drugs induce restriction of 5-HT reuptake (Honig et al., 2009; Rutter et al., 1995). Furthermore, perfusion dialysate contains not only serotonin but also many other small molecules, including other neurotransmitters, which can be collected and analyzed by HPLC and mass spectrometry together with serotonin. Based on this, microdialysis is applied to study the intercorrelations of serotonin and other neurotransmitters, especially dopamine (Bradberry et al., 1993; Perry & Fuller, 1992; Shen et al., 2004), which has a similar chemical structure and interactive function with serotonin. At present, commercially available systems allow long-term and recurrent detection in freely behaving mice: Bjorkli and colleagues detected changes in concentrations of amyloid-β (Aβ) and tau for 18 months, with approximately one measurement per month, in a mouse model of Alzheimer's disease (Bjorkli et al., 2021). Although the targeted chemical is not serotonin, the principle of detection is the same. This application suggests that microdialysis for serotonin detection has the potential for recurrent and long-term detection.

However, despite these advantages, microdialysis suffers from several limitations. One of the significant drawbacks is the poor temporal resolution, limited by passive diffusion of analytes across a semipermeable membrane. The highest temporal resolution for serotonin detection achieved with microdialysis is approximately 1–2 min (Yang et al., 2013; Zhang et al., 2013). In addition, the size of the microdialysis probe determines the low spatial resolution and invasiveness with implantation.

Since microdialysis was advanced for widespread use in the 1980s, it has played a vital role in neurobiology. Along with advanced commercialized systems, many studies use microdialysis to easily detect neurotransmitters, neuromodulators, neuropeptides, and hormones in the brains of behaving animals. In the history of serotonin research, microdialysis has long been the dominant technology for serotonin detection (Figure 2). The popularity of microdialysis provides a great deal of information about the complex serotonergic system.

2.1.2 | Fast-scan cyclic voltammetry

Higher temporal and spatial resolution compared to microdialysis can be achieved with alternative instrumental techniques based on ECD of serotonin in situ in the extracellular space. Electrochemical techniques are based on measuring the current induced by oxidation or reduction of targeted molecules at the electrode surface when applying an electric potential. There are several major variations of electrochemical techniques based on the applied voltage, including amperometry, voltammetry, high-speed chronoamperometry, and FSCV. For example, high-speed chronoamperometry, performed under a constant voltage, is usually employed for mechanistic studies of serotonin release and clearance in vivo (Daws et al., 2005; Daws & Toney, 2007). FSCV, applied with a rapidly cycling voltage, has been particularly popular in the neuroscience field for serotonin monitoring in vivo (Rodeberg et al., 2017; Figures 2 and 4b). Characterized by high temporal resolution and chemical selectivity, FSCV enables

recordings of neurotransmitter level changes on a subsecond timescale. With positively charged groups, monoamines such as dopamine, norepinephrine, and serotonin are attracted to the negatively charged carbon fiber microelectrode surface. Then, they are oxidized and reduced readily by a triangular voltage waveform at a high scan rate (>100 V/s) on the electrode surface (Njagi et al., 2010; Robke et al., 2020). During this chemical reaction, significant currents are generated, which are proportional to the concentration of the neurotransmitters. Thus, this feature enables quantitative analysis of the serotonin concentration. Currents are analyzed by digital means and thus enable direct readout of the release or uptake of neurotransmitters (Dankoski & Mark, 2013).

Fast-scan cyclic voltammetry is orders of magnitude more sensitive than microdialysis. It is often used to monitor acute concentration changes in monoamine neurotransmitters such as dopamine (Park et al., 2011), serotonin (Wood & Hashemi, 2013), and norepinephrine (Park et al., 2011) in freely moving mice. FSCV is also compatible with other sample preparations, such as slices (Abdalla et al., 2020; John & Jones, 2007) as well as primates, including monkeys (Schluter et al., 2014; Yoshimi et al., 2011) and humans (Bennet et al., 2016; Van Gompel et al., 2014). FSCV significantly outperforms microdialysis in terms of temporal resolution; FSCV can record acute and fast fluctuations of serotonin within hundreds of milliseconds (Swamy & Venton, 2007; Wood & Hashemi, 2013). Moreover, the size of an FSCV probe (approximately 6 μm diameter of the tip of electrode; Njagi et al., 2010; Robke et al., 2020) is approximately 30 times smaller than that of a microdialysis probe, thus making FSCV less invasive. For example, FSCV enabled in vivo detection of the rapid release of serotonin in the substantia nigra pars reticulata (SNr) in response to a popular SSRI, escitalopram. The recordings revealed a rapid increase (amplitude corresponding to 13.7 nM) in stimulated serotonin release and a gradual rise in serotonin clearance over 120 min after an acute dose of escitalopram (10 mg/kg) in anesthetized mice (Wood & Hashemi, 2013). Another study applied FSCV to record for the first time two serotonin release events in the mPFC in response to a single stimulation of the medial forebrain bundle, which provides more detailed evidence for understanding serotonin transmission (West et al., 2019).

Nonetheless, there are several limitations on the application of FSCV for serotonin detection in neuroscience. First, because of the similar electrochemical properties of other monoamine neurotransmitters such as dopamine, this method needs to be made more selective for detecting serotonin specifically (Robke et al., 2020). Second, to monitor serotonin concentrations with high sensitivity, it is necessary to reduce the electrode fouling caused by 5-HIAA, the primary metabolite of serotonin. Third, in contrast to the precise concentration in perfusate provided by microdialysis, FSCV provides only a relative change of serotonin level.

As widely used techniques in neuroscience, microdialysis and FSCV have several common advantages, such as high sensitivity, and can be used in freely behaving animals. Furthermore, both microdialysis and FSCV allow measuring 5-HT release and clearance simultaneously, which makes these techniques crucial for studying regulatory mechanisms of the serotonin system (Dankoski & Mark, 2013). However, these probe-based methods are invasive and can cause physical damage to the brain since the probes are implantable detecting equipment in the brain. Moreover, as mentioned above, serotonin neurons are mainly located in the DRN (Liu et al., 2014), a problematic region for implanting devices. Because of the size of the probes, the spatial resolution of these two methods is also limited. For FSCV, the spatial resolution is approximately less than 10 μm (Bass et al., 2010; Maina et al., 2012). They cannot provide details such as subcellular spatial resolution and cell types within recorded brain regions. The two types of serotonin transmission can provide insight into the extracellular serotonin transients of volume transmission and the receptor framework surrounding the electrode rather than fast synaptic transmission.

2.2 | Tomography: fMRI and PET

The non-imaging methods discussed above, such as microdialysis and FSCV, provide powerful tools to study serotonin function in small mammals. However, they are not practical for general use in humans because of their invasiveness. Tomography approaches such as fMRI and PET offer a minimally invasive detection method (invasiveness mainly originates from the need to inject contrast agents and radioligands into bloodstream) and a general serotonin kinetics picture against point detection methods such as microdialysis and FSCV that measure changes in serotonin levels around the probe. It provides neural activity mapping of more extensive regions in the brain or even the whole brain.

2.2.1 | fMRI: BOLD combined with ATD and fMRI probes

The primarily used fMRI is blood oxygen level-dependent (BOLD) imaging, which was discovered by Ogawa et al. (1990). It is based on hemoglobin, an endogenous oxygen-sensitive MRI contrast agent existing in red blood cells. When neurons become active, they consume more energy and need blood flow to bring more oxygenated blood to displace deoxygenated blood. Oxygenated hemoglobin is diamagnetic, a term that describes that it is virtually resistant to magnetism. Compared to oxygenated hemoglobin, however, deoxygenated hemoglobin is paramagnetic, which can be exploited by fMRI for signal detection. Because of the general distribution of blood vessels and the complexity of neurovascular coupling, BOLD fMRI provides only a brain-wide non-specific readout of neural activity. Thus, to track serotonergic activity, researchers combine BOLD with acute tryptophan depletion (ATD), a dietary technique to reduce levels of tryptophan and then reduce serotonin levels in a subject (Young, 2013; Figure 5a). Typically, Trp is a dietary serotonin precursor and an essential amino acid that is transported across the BBB by a carrier protein (Mehedint & Gulledge, 2014). To implement ATD, subjects adopt a Trp-depleted diet that is otherwise balanced

for other essential amino acids, and this leads to a sharp drop in Trp when measured in their blood plasma levels. As such, low Trp levels in blood plasma lead to reduced availability of Trp in the brain and a decrease in serotonin levels. In this physiological context, by comparing the difference in neural activity captured by BOLD between the normal state and the state after ATD, the influence of reduced serotonin is established.

The combination of ATD and fMRI provides an approach to assessing the brain's behavioral effects of reduced serotonin metabolism. For example, the application of ATD combined with BOLD fMRI verified that serotonin significantly influences PFC–amygdala circuits implicated in aggression and other affective behaviors of humans (Passamonti et al., 2012). Other studies have shown the effect of ATD on human face activation processing, as ATD attenuated activation in areas related to emotion processing (Fusar-Poli et al., 2007; van der Veen et al., 2006). Less commonly, some research has applied ATD combined with PET (da Cunha-Bang et al., 2019) and microdialysis (van der Plasse et al., 2007). Although manipulating the level of 5-HT through ATD provides an approach to assess the behavioral effects of reduced serotonin metabolism in the brain, the low spatiotemporal resolution of this method limits its further application in practical research. BOLD fMRI combined with ATD only provides an indirect readout of neural activity after serotonin levels decrease. Moreover, the underlying mechanism for ATD remains unclear, and the utility of this method remains controversial (van Donkelaar et al., 2011).

Since the discovery of BOLD signaling, attempts have been made to improve the signal-to-noise ratio of fMRI. Considerably more precise detection would be achieved by fMRI sensors that directly measure serotonin dynamics. Scientists aimed at proteins that are paramagnetic and show ligand-binding or catalytic properties. For example, a fatty acid hydroxylase called flavocytochrome P450-BM3 (BM3) caught the attention of researchers. BM3 contains a paramagnetic iron atom embedded into a solvent-accessible substrate-binding pocket. Binding of ligands to this pocket will lead to a drop in MRI signal and a shift in optical absorbance, which works as the readout of ligand level change. Based on the genetically encoded nature of metalloproteins, several specific fMRI probes have been developed based on paramagnetic proteins that target ions and chemicals involved in neuroactivity, including calcium (Barandov et al., 2019), dopamine (Shapiro et al., 2010), and serotonin (Brustad et al., 2012).

Called BM3h-2G9C6 (Figure 5a), this fMRI sensor for 5-HT performs with a submicromolar affinity ($K_d = 0.7 \pm 0.2\,\mu M$) and is over 250-fold more selective over dopamine and norepinephrine ($K_d = 198 \pm 7\,\mu M$ and $K_d = 275 \pm 40\,\mu M$, respectively; Brustad et al., 2012). This probe has been effective in recording decreases in MRI contrast with comparable ligand-free relaxivity (r1), a parameter used to evaluate the strength of a contrast agent (Brustad et al., 2012). Thus, with this 5-HT MRI contrast agent, researchers built three-dimensional (3D) functional imaging of serotonin transport in vivo by directly injecting the BM3h-2G9C6 complex with 5-HT and detecting the disassociation activity and reuptake of released serotonin by endogenous proteins in the brain. This approach revealed widespread non-saturating serotonin removal by SERT on the cells of anesthetized rats, with maximal rates detected in the lateral septum (Hai et al., 2016). Currently, while this paramagnetic probe provides higher spatiotemporal resolution than BOLD, its application has thus far been reported only in studies with rodents. Additionally, the use of these paramagnetic probes invariably requires them to be injected as chemical cocktails at high concentrations into subjects, and it is currently suitable only for studying 5-HT clearance mechanisms. Additionally, continuous injection is currently not feasible, and fMRI is limited

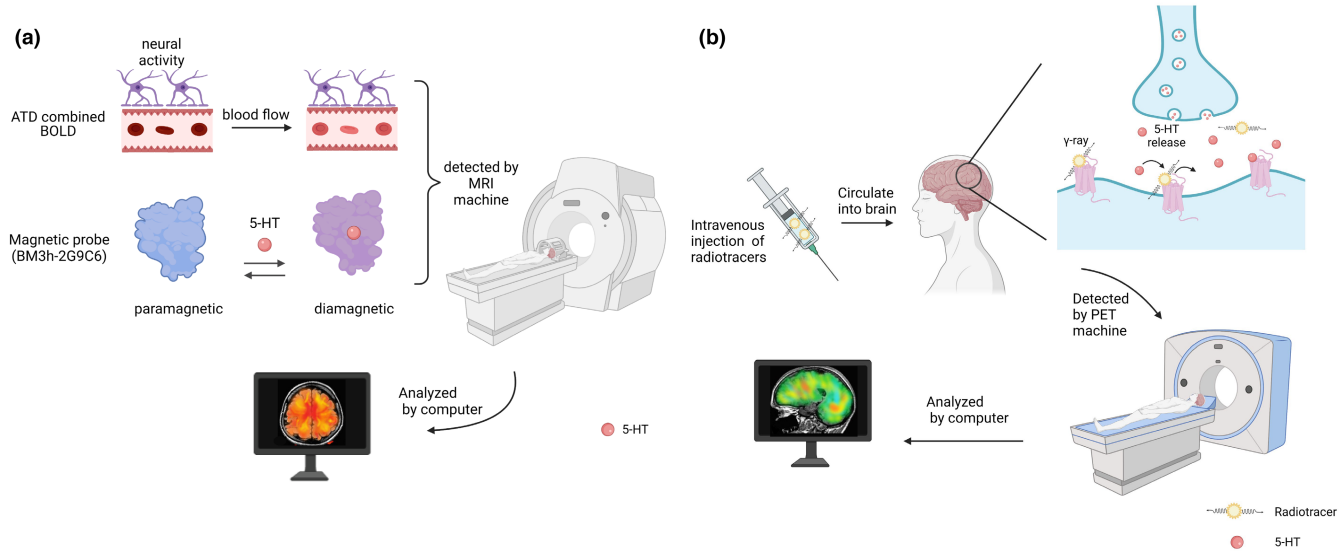

FIGURE 5 Schematic representation of tomographic methods for serotonin analysis in vivo. (a) BOLD fMRI coupled with ATD and paramagnetic probe fMRI. (b) Radiotracer-targeted PET for serotonin detection. 5-HT, 5-hydroxytryptamine; ATD, acute tryptophan depletion; BOLD, blood oxygen level-dependent; fMRI, functional magnetic resonance imaging; PET, positron emission tomography.

for behavioral studies that require even relatively small limb movements.

2.2.2 | PET

Positron emission tomography neuroimaging is a powerful method that employs radioactive tracers for functional imaging of physiological activities and metabolic processes in vivo (Figure 5b). Radioactive tracers consist of molecules incorporating a radioactive atom called a radioisotope, such as [^{11}C] and [^{18}F]. These molecules are specifically designed and synthesized to bind targeted proteins or work as substrates in the body. After synthesis, isotope-labeled tracers are usually administered to the test organism through intravenous injection. After passing through the BBB, radioactive tracers bind to targeted proteins or become involved in specific metabolic processes in the brain. The isotope can emit γ-rays continuously, working as the readout of targeted protein activity or metabolic processes. The emission process is called annihilation: the positron from the radioactive isotope encounters the nearby electron at the right speed and annihilates with each other, producing γ-rays emitted in two opposite directions. The PET detector machine, in the shape of a ring surrounding the testing object, detects and records the γ-ray signal. All data collected in the PET scanner are analyzed by a computer to localize the signal source and concatenated spatially to reconstruct a full 3-dimensional map of neural activities. The tracers spread over volumes of tens of microliters through convective forces, permitting kinetic measurements over the entire brain at different time points.

Depending on the different purposes of PET imaging, different tracers are selected and used. For example, [^{18}F]fluorodeoxyglucose, a glucose analog, is commonly used for cancer tracing. Increased uptake of [^{18}F]fluorodeoxyglucose reflects increased metabolic activity, which is closely correlated with cancer cells. For PET imaging of the serotonergic system, [^{11}C] and [^{18}F] are the predominant isotopes used to label synthetic molecules as ligands for serotonin receptors, SERT, and substrates for serotonin metabolism (Beliveau et al., 2020; da Cunha-Bang et al., 2019; Silberbauer et al., 2020). Radiotracers that have high affinity and selectivity for a given target are selected and identified. To date, multiple synthetic ligands have been designed and applied to specifically target SERT or serotonin receptors (Huang et al., 2010). For example, the radiotracer [^{11}C]DASB is a radioligand with specificity and selectivity for binding SERT. When serotonin is absent, the radioactive tracers bind to SERT with a radioactive signal. After serotonin release, since the affinity of serotonin is better than that of [^{11}C]DASB, it efficiently competes with [^{11}C]DASB for protein binding, such that native serotonin binding to SERT clears the isotope-labeled tracer from the binding position. This fluctuation of the isotope-labeled binding signal, correlated with the reuptake of serotonin, is then detected by a PET scanner and analyzed to quantify serotonergic activity. Through this approach, the high selectivity of the existing tracers allows specific targeting of serotonin receptor subtypes (Colom et al., 2019; Saulin et al., 2012). For example, validated ligands such as [^{18}F]altanserin are aimed at the 5-HT$_{2A}$ receptor, and [^{11}C]CUMI-101 is aimed at the 5-HT$_{1A}$ receptor (Saulin et al., 2012). This type of tracer provides a tool for researching the distribution and function of specific subtypes of 5-HT receptors in different brain regions. In addition, the synthesis of serotonin can be traced by isotope-labeled 5-HTP, a metabolic intermediate in the biosynthesis of 5-HT (Figure 3a; Saulin et al., 2012). The same is true for the degradation of serotonin through the high selectivity of the tracer for MAO (Saulin et al., 2012). This unique property sets PET aside from all other methods for serotonin detection, as it enables targeting subtypes of serotonin receptors and specific serotonergic pathways.

Neuroimaging using PET provides us with the unique opportunity to quantify serotonergic activity in healthy and diseased human brains in vivo. For example, the demonstration of reduced 5-HT$_{1A}$ receptor binding in social anxiety disorder patients is revealed through PET (Lanzenberger et al., 2010; Spindelegger et al., 2009). Using [^{11}C]-labeled SERT ligands, such as [^{11}C]DASB, a reduction in SERT binding appeared in ecstasy (MDMA, a hallucinogenic drug that dramatically enhances serotonin activity) users, which reflects an increase in presynaptic serotonin reuptake (McCann et al., 2005). When [^{11}C]5-HTP and [^{11}C]DASB are applied independently, neurotransmission in social anxiety disorder is marked by an overactive presynaptic serotonin system (Frick et al., 2015). This overactivity is coupled with increased serotonin synthesis and transporter availability. The physiological mechanism revealed by PET imaging provides critical new insights into the etiology of social anxiety disorder and other diseases.

While PET imaging is widespread and highly informative for human studies, there are several limitations in its application for neuroscience. For example, the synthesis of radioactive tracers and detection of PET signals altogether require specific and expensive equipments, making it inconvenient for most laboratories that study serotonergic activity. Additionally, radiotracer isotopes are unstable and have a specific half-life, which is a term that defines the amount of time it takes for an isotope to lose half of its radioactivity. [^{18}F] and [^{11}C], the predominant isotopes used in tracers to study 5-HT, have half-life of approximately 109 min ([^{18}F]) and 20 min ([^{11}C]), respectively, (Mahony & Markowitz, 1964; Tu & Mach, 2010), which means that tracers must be transported, injected, and detected promptly after their synthesis. For the subject, exposure to radioactivity also poses a potential risk to health, however insignificant this may be. Furthermore, distinguishing between small changes in targeted protein density or changes in 5-HT occupancy of target protein is challenging for signals resolved from PET (Placzek et al., 2016). These represent several issues for PET imaging that must be considered when studying serotonin signaling in health and disease.

In comparison to microdialysis and FSCV, a significant advantage offered by imaging techniques such as BOLD fMRI and PET is their minimal invasiveness, which explains their broad use in clinical research studies, such as studies on the molecular mechanisms of disease in humans. However, tomography detection is of a specific, defined resolution both spatially (micrometers to millimeters) and temporally (on the scale of seconds). Notably, fMRI probes and PET

tracers take several minutes to circulate across the body to be useful to detect 5-HT in the brain. Because of its limited temporal resolution, PET is not suitable for detecting fast synaptic transmission, but rather, it is used for slow serotonergic activity imaging over large brain regions. Moreover, regarding detection requirements, both fMRI and PET require sophisticated equipment and specific conditions for scanning, including a requirement that the subject lies flat and relatively motionless for data collection. Tomography detection approaches represent a limitation to tracing serotonergic activity in the context of awake behaviors, even for observations requiring relatively minor limb movements. Remarkably, for fMRI probes and PET tracers, although implanted devices are not needed, the procedures still require intravenous injection, typically of high concentrations of exogenous agents, such as radiotracers or paramagnetic sensors, into the subject, which may influence regular physiological activity. Thus, it is clear that these methods do not overcome the aforementioned detection challenges, and better in vivo serotonin monitoring methods are needed.

3 | FLUORESCENCE IMAGING METHODS: GENETICALLY ENCODED FLUORESCENT SENSORS

As fluorescence microscopy has been developed with improved imaging depth, spatiotemporal resolution, and relatively accessible equipment for bioimaging, many fluorescence probes have emerged for real-time recordings of serotonin in living cells and organisms.

In recent decades, multiphoton microscopy has improved the ability to measure small quantities of biological molecules. Three-photon microscopy was used to image the 5-HT concentration and content of individual secretory granules in rat basophilic leukemia cells under an excitation wavelength of 700 nm (Maiti et al., 1997). It was also applied for imaging somatic serotonin release in a quantitative manner in serotonergic neurons and brain slices (Kaushalya et al., 2008). Although serotonin can be directly observed by multiphoton microscopy, the achievable signal-to-noise ratio (SNR) and temporal resolution are suboptimal for in vivo measurements, precluding the widespread application of label-free serotonin two-photon imaging.

As fluorescence imaging is a well-developed approach that is essential to solving biological questions, many fluorescent probes have been optimized for measuring serotonin in recent decades. For example, a fluorescent false neurotransmitter (FFN246; Henke et al., 2018) specifically binds to SERT and VMAT with fluorescent signals, enabling optical measurement of serotonin kinetics. Nevertheless, this tracer needs to be delivered to sites of interest for labeling serotonin, such as through exposure to media applied to cultured cells and brain slices, and this limits its wider application. In contrast, cell-based neurotransmitter fluorescent-engineered reporters (CNiFERs) are engineered cells expressing a chosen receptor combined with a fluorescent calcium sensor. It utilizes the receptor cascade to transform receptor activity into a rise in cytosolic [Ca^{2+}] levels and reports [Ca^{2+}] signals by a fluorescent calcium sensor. For instance, 5-HT_{3A}-based CNiFER combines the 5-HT_{3A} receptor, a ligand-gated ion channel, with a fluorescent calcium sensor (Yamauchi et al., 2011). After serotonin binds to the 5-HT_{3A} receptor, the opening of the ion channel leads to calcium influx, inducing calcium fluorescent sensor (TN-XXL) signal changes. However, this type of fluorescent technique indirectly reports serotonin release and the temporal resolution of the reporter signal is a significant factor that needs to be considered when applied to behavioral studies. Another signaling pathway-based assay (Tango assay) transmits the activation of the 5-HT_{2C} receptor to downstream EGFP expression through transcription factors (Watanabe et al., 2016). This assay provides a single-cell resolution, but the temporal resolution is on the scale of tens of hours, limiting further application. Many tools based on fluorescence have enriched the toolbox for measuring serotonin dynamics. However, these resources have not been widely deployed for biological studies of serotonin homeostasis because of multiple drawbacks and limitations. Because of poor performance for serotonin dynamics detection in vivo, they have not been adopted by neuroscientists; therefore, we will not discuss them further in this review.

Recently, genetically encoded serotonin indicators (GESIs) have been developed for direct recurrent 5-HT measurement in vivo, such as iSeroSnFR (Unger et al., 2020), $GRAB_{5\text{-}HT}$ (Wan et al., 2021), psychLight (Dong et al., 2021), and sDarkern (Kubitschke et al., 2022). They can provide release dynamics at a subsecond resolution, with submicromolar affinity and high molecular specificity. Here, we focus on the development and properties of GESIs in vivo.

3.1 | Design of genetically encoded sensors

In the past 10 years, the power of fluorescent proteins (FPs) and their numerous derivatives has been harnessed for the development of genetically encoded fluorescent biosensors for detecting neuronal activity (Lin & Schnitzer, 2016; Piatkevich et al., 2019). To date, more than 100 different genetically encoded biosensors have been advanced for detecting physiological ions (Greenwald et al., 2018), membrane potential (Knöpfel & Song, 2019), neurotransmitters (Lin & Schnitzer, 2016; Piatkevich et al., 2019; Sabatini & Tian, 2020), and signaling pathways (Greenwald et al., 2018; Zhou et al., 2020). The developed sensors provide a window into the real-time biochemistry of living cells and organisms such as *Drosophila* (Keller et al., 2021; Wan et al., 2021), *Caenorhabditis elegans* (Borden et al., 2020), zebrafish (Keller et al., 2021; Sun et al., 2018), mice, rats (Szebényi et al., 2015; Zhao & Braunewell, 2008), and monkeys (Bollimunta et al., 2021; Saunders et al., 1994).

Genetically encoded fluorescent indicators for neurotransmitters are typically comprised of two parts: a reporter moiety consisting of FP(s) and a sensing moiety represented by a neurotransmitter binding domain. The principle of the sensor design is based on the conformational changes in the sensing moiety induced by specific ligand binding. This conformational change is transduced to the

reporter domain, resulting in an increase or decrease in the fluorescence intensity.

For the reporter domain, two molecular designs have been successfully employed for the development of genetically encoded fluorescent indicators. One type is based on Förster resonance energy transfer (FRET), which has two FPs of overlapping emission and excitation spectra. The conformational change in the binding domain leads the distance between the donor FP and acceptor FP to decrease to a specific range, which induces FRET and provides a specific fluorescent signal (Lindenburg & Merkx, 2014). The first serotonin sensor, 5-HT-CC, is a FRET-based genetically encoded sensor that is engineered by inserting Citrine into the third intracellular loop of the 5-HT_{1B} receptor and adding Cerulean at the C-terminus (Candelario & Chachisvilis, 2012). This sensor shows an approximately 4% FRET ratio change at a saturated 5-HT concentration with a half-maximal effective concentration (EC_{50} or K_d) of approximately 480 nM. The modest fluorescence response and the fact that they have been validated only in cultured cells (HEK cells and MC3T3 cells) have hampered their practical application in vivo. The other type is based on a single FP; in this case, the conformational change in the sensing domain alters the chromophore environment of the FP, leading to an increase or decrease in the fluorescence intensity. Compared to FRET-based sensors, constructs utilizing a single FP design typically have a broader fluorescent dynamic range and higher SNR.

Binding elements are usually classified into two main classes by ligand-binding scaffolds: bacterial periplasmic binding proteins (PBPs) and GPCRs. We will discuss these two classes below in detail.

3.2 | PBP-based serotonin sensor

Natural bacterial PBPs can bind to many small molecules and peptides. Binding to a ligand induces conserved conformational changes in PBPs, and this molecular feature can be customized through directed molecular evolution to engineer synthetic PBPs that bind ligands of choice, such as neurotransmitters. Based on this, many biosensors for neurotransmitters and other physiologically important small molecules, such as glutamate (Marvin et al., 2013), GABA (Marvin et al., 2019), glucose (Keller et al., 2021), and acetylcholine (Borden et al., 2020), have been developed. Because of the properties of PBPs, they can naturally bind with neurotransmitters and chemicals with comparable affinity, which speaks to the attractiveness of this approach to developing novel sensors.

The first PBP-based sensor for detecting 5-HT, iSeroSnFR, is constructed from iAChSnFR0.6, an early version of the PBP-based sensor for acetylcholine (Figure 6a; Unger et al., 2020). Since there was no annotated PBP for 5-HT and iAChSnFR0.6 exhibits a binding affinity for serotonin (apparent K_d >1 mM; Borden et al., 2020), Unger et al. redesigned the binding pocket of iAChSnFR0.6 guided by computational design and machine learning to improve protein–ligand binding specificity and affinity for serotonin (Unger et al., 2020). After three rounds of iterative mutagenesis and screening for improved affinity to serotonin and the fluorescence dynamic range, the final version of iSeroSnFR was advanced with higher affinity (K_d = 390 μM in HEK cells) as well as high temporal resolution in the purified protein version (Table 2). Compared to iAChSnFR0.6, iSeroSnFR contains 19 mutations in total, and it has more than a 5000-fold improvement in 5-HT affinity while eliminating choline and acetylcholine binding. Indeed, the affinity of iSeroSnFR for serotonin is found to be 18-fold greater than its affinity for dopamine (as calculated from the data shown in Supplement of Unger et al., 2020). It shows a maximal 17-fold fluorescence increase ($\Delta F/F_0$, maximum change in fluorescence signal from basal level of serotonin-free state) upon 5-HT binding in HEK cells when expressed on the extracellular surface of the plasma membrane using the pDisplay system.

For applications in vivo, iSeroSnFR is fused with membrane localization motifs to enable detection of extracellular serotonin. A recent study confirmed that the iSeroSnFR fluorescence signal measured using averaged trials correlated well with electroencephalography and electromyography measurements in the basolateral amygdala of mice across the sleep/wake cycle (Unger et al., 2020). Additionally, Hon and colleagues applied iSeroSnFR to investigate 5-HT release in the context of fear learning and recall (Hon et al., 2022). Notably, the fluorescent dynamics of this sensor in brain slices (the maximal $\Delta F/F_0$ is approximately 12%) and with freely moving mice (the maximal $\Delta F/F_0$ is approximately 12% during the sleep/wake cycle) are significantly low in both contexts. The poor in vivo performance of iSeroSnFR can be attributed to the difference between the K_d of iSeroSnFR and the physiological range of extracellular serotonin concentrations in the brain. Therefore, it is crucial to optimize the K_d of sensors to match the physiological range in the brain as closely as possible.

Since the sensor iSeroSnFR is a water-soluble protein, Unger et al. used it in cultured cells as an intracellular probe to measure serotonin transport via functionally characterized human SERT expressed in HEK cells. In their study, they reported that iSeroSnFR was effective for measuring time-resolved kinetics of 5-HT influx and efflux and transport modulation by various drugs (Unger et al., 2020). Because iSeroSnFR does not respond to most clinical drugs, it is an ideal assay for studying the interactions between serotonin-related proteins and drugs and facilitating drug discovery. However, water solubility is a double-edged sword in that it means adding an additional membrane-localized signal, which may influence protein properties or structure.

Evolved from the early, engineered versions of the acetylcholine biosensor, iSeroSnFR has a wide fluorescence dynamic range for serotonin detection, while improvements to its affinity would be worthwhile to further enhance it for measuring physiological concentrations of serotonin with greater accuracy, such as what is reported (Table 2) for GPCR-based serotonin sensors that have a superior K_d for serotonin binding. This is discussed further below.

3.3 | GPCR-based serotonin sensors

Most neurotransmitters and neuromodulators express GPCRs as their native binding proteins. Taking this fact as an advantage, a

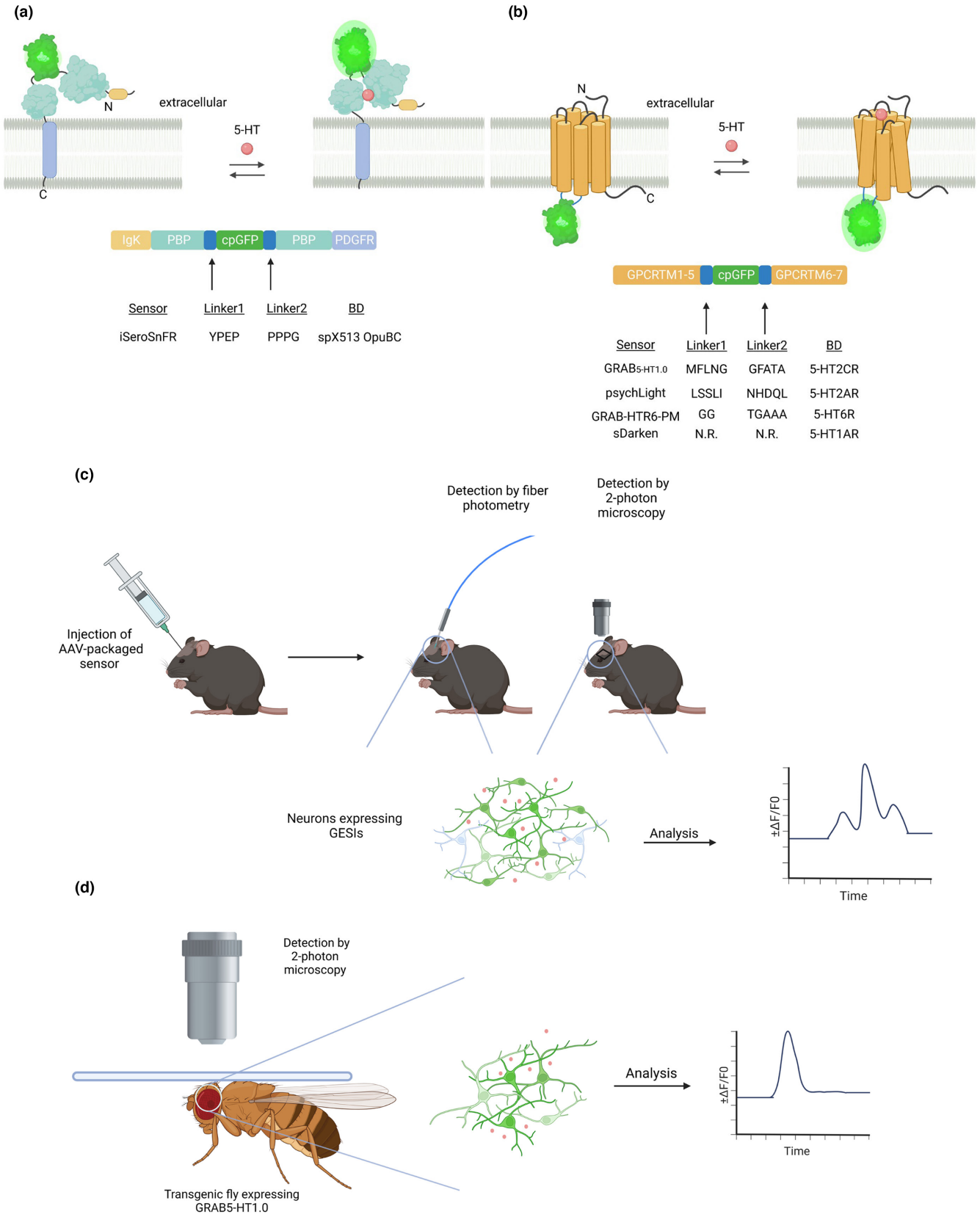

FIGURE 6 Imaging of serotonin in vivo using GESIs. (a) Molecular design of iSeroSnFR. (b) Molecular design of GPCR-based serotonin biosensors. (c) Serotonin imaging in mice using two-photon microscopy or fiber photometry. (d) Serotonin imaging in transgenic flies using two-photon microscopy. 5-HT, 5-hydroxytryptamine; BD, binding domain; GESIs, genetically encoded serotonin indicators; GPCRs, G protein-coupled receptors; N.R., not reported.

TABLE 2 Properties of genetically encoded fluorescent sensors.

Name	Molecular scaffold	Ex/Em	EC50	$\Delta F/F_0$ (%)	τ_{on} (ms)	τ_{off} (ms)	Application in vivo: organism(s)	Reference
iSeroSnFR	PBP(spX513 OpuBC)	Green	390 μM[a]	1700[a]	fast: 0.5-10[b] slow: 5000–18 000[b]	Fast: 4[c] Slow: 150[c]	Mouse	Unger et al. (2020)
psychLight	GPCR(5-HT2A)	Green	26.3 nM[a]	79.60[a]	NA	NA	Mouse	Dong et al. (2021)
$GRAB_{5\text{-}HT1.0}$	GPCR(5-HT2C)	490/510	22 nM[c]	250[a]/280[c]	200[a]	3130[a]	Fly, mouse	Wan et al. (2021)
GRAB-HTR6-PM	GPCR(5-HT6)	Green	84 nM[a]/28 nM[c]	~150[a]/~40[c]	NA	NA	–	Sheu et al. (2021)
sDarken	GPCR(5-HT1A)	Green	127 nM[a]	~64[a]	43.5[a]	323[a]	Mouse	Kubitschke et al. (2022)
H-sDarken	GPCR(5-HT1A)	Green	57 nM[a]	~−68[a]	57.2[a]	324.8[a]	–	Kubitschke et al. (2022)
L-sDarken	GPCR(5-HT1A)	Green	145 μM[a]	~−70[a]	95.3[a]	156.0[a]	–	Kubitschke et al. (2022)

Abbreviations: τ_{off}, time required to return to the basal state from the maximum response; τ_{on}, time required to reach the maximum response; EC_{50}, half-maximal effective concentration; Em, emission wavelength; Ex, excitation wavelength; $\Delta F/F_0$, maximum change in fluorescence signal from the basal state to the bound state.

[a]Expressed in HEK293 cells.

[b]Tested on the purified protein form.

[c]Expressed in dissociated neurons.

series of genetically encoded fluorescent indicators for neurotransmitters and neuromodulators based on GPCRs have been developed (Lin & Schnitzer, 2016; Sabatini & Tian, 2020). As ancient proteins, GPCRs share a highly conserved structural topology comprising seven α-helix transmembrane domains (TMs), three intracellular loops, and three extracellular loops (Palczewski et al., 2000; Rosenbaum et al., 2009). Structural studies indicate that ligand binding induces the largest conformational changes in GPCRs by causing a shift of up to 6 Å between TM5 and TM6 (Dalton et al., 2015; Lu et al., 2021; Park et al., 2008; Scheerer et al., 2008). For this reason, GPCR-based sensors are engineered by inserting the third intracellular loop between TM5 and TM6 with circularly permuted GFP (cpGFP). This molecular design strategy has been effective in constructing GPCR-based sensors for dopamine (Sun et al., 2018), acetylcholine (Jing et al., 2020), endocannabinoids (Dong et al., 2020), ATP (Wu et al., 2022), norepinephrine (Feng et al., 2019), and serotonin, demonstrating the versatility of the approach.

The first working prototype of a GPCR-based serotonin sensor was developed by inserting cpGFP into the third intracellular loop of the $5\text{-}HT_{2A}$ receptor (Patriarchi et al., 2018). Further enhancement of the fluorescence dynamic range via linker optimization and site-directed mutagenesis resulted in the next-generation indicator psychLight1 (Dong et al., 2021; Figure 6b). Further modification of psychLight1 through introduction of an endoplasmic reticulum export motif resulted in the psychLight2 variant with improved membrane expression, which was used for subsequent applications in vivo (Dong et al., 2021). The psychLight1 indicator shows approximately 79.6% $\Delta F/F_0$ maximally characterized by an EC_{50} value of 26.3 nM in HEK cells (Table 2; Dong et al., 2021). Although the dynamic range of psychLight1 is relatively limited compared to those of many other next-generation neurotransmitter sensors (Sabatini & Tian, 2020), it has advantages for practical applications such as drug screening. For example, psychLight1 maintains the intrinsic specificity of the $5\text{-}HT_{2A}$ receptor toward ligands such as classic hallucinogens, atypical antipsychotics, and psychoplastogens. Therefore, psychLight1 can be used to screen the hallucinogenic effects of $5\text{-}HT_{2A}$ receptor agonists by testing responses to different drugs. Drug screening of psychLight2 in cultured cells identified a non-hallucinogenic psychedelic analog that produced rapid-onset and long-lasting effects resembling those of antidepressants after a single administration (Dong et al., 2021). Despite potential application in drug discovery, no further verification of sensor properties, such as kinetic and specificity measurements, was performed for psychLights. Furthermore, the performance of psychLight2 in behaving mice using fiber photometry was not sufficient for single-trial imaging of serotonin, which is crucial for behavioral studies. To make it practical for single-trial in vivo imaging, the maximum change in the fluorescence intensity of psychLights from the basal state to the binding state should be improved in the future.

Another GPCR-based sensor for 5-HT is $GRAB_{5\text{-}HT1.0}$ (Figure 6b), which was developed by inserting cpGFP into the $5\text{-}HT_{2C}$ receptor (Wan et al., 2021). $GRAB_{5\text{-}HT1.0}$ was validated in cultured cells, mouse brain slices, and living fly and mouse brains. It shows a higher

affinity for serotonin (EC_{50} = 22 nM in neurons) and higher specificity for serotonin against dopamine (approximately 31-fold) and norepinephrine (approximately 36-fold) than iSeroSnFR (Wan et al., 2021; Table 2). $GRAB_{5\text{-}HT1.0}$ exhibits up to an approximately threefold fluorescence increase ($\Delta F/F_0$) in cultured neurons upon saturating administration of exogenous 5-HT. Regarding the sensor's kinetics, the τ_{on} and τ_{off} values (tested in cell culture) are hundreds of times slower than iSeroSnFR (measured in solution), approximately 0.2 and 3.1 s, respectively, which restricts the detection of fast 5-HT dynamics (Table 2). Since GPCRs have downstream signaling, which may potentially interrupt normal physiological cell activity, it is essential to verify the signaling pathway of $GRAB_{5\text{-}HT1.0}$. As measured by calcium imaging, cAMP imaging, G protein-dependent luciferase complementation assays, Tango assays, and long-term measurements, $GRAB_{5\text{-}HT1.0}$, unlike the native $5\text{-}HT_{2C}$ receptor, showed no detectable coupling to downstream signaling pathways, indicating the safety of the sensor in cells (Wan et al., 2021). Concurrent fluorescence imaging and FSCV recording of DRN in acute brain slices demonstrated that $GRAB_{5\text{-}HT1.0}$ had approximately twice the SNR of FSCV. As verified in a transgenic *Drosophila* model using two-photon microscopy, the $GRAB_{5\text{-}HT1.0}$ sensor in either serotonergic dorsal paired medial (DPM) neurons or Kenyon cells (KCs) could report 5-HT release in the β' lobe of the mushroom body (MB) in response to odor application or body shock, consistent with a previous physiological study in the DPM (Wan et al., 2021; Zeng et al., 2022). The $GRAB_{5\text{-}HT1.0}$ biosensor showed approximately 50% $\Delta F/F_0$ in acute brain slices when given 100 pulses of stimuli at 100 Hz. During the sleep–wake cycle, the fluorescence signal changes are consistent with electroencephalography and electromyography recordings in the basal forebrain, the orbital frontal cortex, and the bed nucleus of the stria terminalis when measured in freely behaving mice using fiber photometry. Flanigan et al. employed in vivo fiber photometry with the $GRAB_{5\text{-}HT1.0}$ serotonin sensor to identify neurons that sex-specifically coregulate affective behaviors and are modulated by binge alcohol consumption (Flanigan et al., 2022). Under two-photon microscopy, the fluorescence changes maximally up to approximately 55% in behaving mice after injection of MDMA, which increases the serotonin level (Wan et al., 2021). However, the in vivo performance of $GRAB_{5\text{-}HT1.0}$ is still significantly lower than that of well-established green calcium biosensors, such as the GCaMP6 series. Compared to the dynamic range observed in cell culture, $GRAB_{5\text{-}HT1.0}$ exhibited a significantly limited fluorescent signal response in vivo. For future applications in animals for chronic imaging, the dynamic range and kinetics of $GRAB_{5\text{-}HT1.0}$ should be further enhanced.

It is recognized that different subtypes of serotonin GPCRs signal uniquely to give rise to distinct physiological effects. As such, a variety of designs incorporating distinct scaffolds of GPCR molecules have been used to develop a range of serotonin sensors. For example, a cilia-targeted serotonin sensor (GRAB-HTR6-PM) based on the $5\text{-}HT_6$ receptor was developed to observe serotonin release onto cilia by activation of serotonergic axons (Sheu et al., 2021). The sensor was validated in HEK cells, and HaloTag was added to improve the sensor for visualizing cilia (HTR6-GRAB-cilia). Although the affinity of the cilia-targeted HTR6-GRAB-cilia sensor is high (28 nM), one major limitation of its use for in vivo imaging is its low reporter signal change (up to 40%).

Recently, a new family of GESIs (sDarken) was developed based on the $5\text{-}HT_{1A}$ receptor (Kubitschke et al., 2022). In contrast to all other GESIs, *sDarken* 5-HT biosensors are a set of turn-off sensors that exhibit bright baseline fluorescence when 5-HT is absent and diminish their fluorescence upon binding. *sDarken* shows a high affinity of approximately 127 nM and faster kinetics than the GRAB 5-HT sensor (Table 2). Two versions of sDarken (L-sDarken and H-sDarken) were developed with 5-HT affinities that span more than 3 orders of magnitude ranging from 56 nM to 145 μM while maintaining fast kinetics of the parental sDarken. Validated in the PFC of mice by 2-photon imaging, sDarken provided tracing for serotonin dynamics triggered by electrical stimulation. However, this negative response type of sensor has a low SNR and might not be ideal for in vivo imaging.

3.4 | Advantages and disadvantages of GESIs

Compared to non-optical methods and small molecular probes, GESIs have superior spatiotemporal resolution, sensitivity, and specificity (Table 3). One main advantage of GESIs is that they are genetically encoded, which means that delivery of this 5-HT biosensor into brain tissue occurs via gene transduction and with the capability to target the sensor to specific cell types. For example, GESIs are useful for studying neurotransmitter signaling in small model organisms such as *Drosophila* via a transgenic approach, for which analytical methods and tomography are not practical (Wan et al., 2021). Furthermore, this allows optimization of the biosensors via protein engineering approaches such as directed evolution. At present, the combination of different approaches, including genome mining, machine learning, and computational design, is providing new avenues for sensor development and optimization, which has already resulted in a great diversity of GESIs (Table 2). It is worthwhile to mention a creative attempt to use tick lipocalin as a scaffold to develop a fast and 5-HT-specific sensor called G-GESS (Zhang et al., 2020). This work opens a new door for exploring novel scaffolds as binding elements of sensors through genome mining. Another beneficial property of GESIs is their relatively compact coding sequence, with iSeroSnFR encoded by an approximately 1.5 kb cDNA sequence (Unger et al., 2020), while cDNAs for psychLights (Dong et al., 2021) and $GRAB_{5\text{-}HT1.0}$ (Wan et al., 2021) are approximately 2.1 and 2 kb in length, respectively. Their compact sequences afford efficient packaging into viral vectors used in neuroscience for gene expression in specific brain regions, such as AAVs. Through delivery with AAVs, the expression of GESIs in vivo is evident for several months (Dong et al., 2021; Unger et al., 2020; Wan et al., 2021), which makes this approach highly attractive for studying chronic behaviors and patterns of neuron activity. Furthermore, the biosensors can be selectively expressed in certain cell types using Cre-driver lines and

TABLE 3 State-of-the-art serotonin detection methods.

Method	Description	Target	Advantages	Disadvantages	Reference
Microdialysis	A microdialysis probe with a semipermeable membrane at the tip is surgically implanted into the brain, and analytes are collected over time	5-HT in extracellular dialysate analyzed by MS or spectroscopy	High specificity and sensitivity; suitability for long-term behavioral study; repeatability	Poor spatiotemporal resolution (temporal resolution is approximately 1–2 min); invasiveness; lack of real-time monitoring	Yang et al. (2013), Zhang et al. (2013)
FSCV	Carbon-fiber microelectrodes detect currents originating from the oxidation of 5-HT on the probe surface to reflect 5-HT dynamics	Currents generated by oxidation of extracellular 5-HT attached on the electrode	High temporal resolution (millisecond timescale)	Poor spatial resolution; invasiveness	Swamy and Venton (2007), Wood and Hashemi (2013)
fMRI	Brain activity is mapped and measured based on blood flow or exogenous MRI-active probes	Hemoglobin or magnetic probe specific to 5-HT	Minimally invasive; 3D whole-brain imaging	High cost; poor spatial and temporal resolution (on the order of micrometers and seconds, respectively); dependence on a special environment	Fusar-Poli et al. (2007), Brustad et al. (2012)
PET	Positron-emitting ligands target specific 5-HT-related proteins and metabolites to monitor serotonergic activity	Activity of specific 5-HT-related proteins and metabolites	Minimally invasive; 3D whole-brain imaging; sensitivity	High cost; poor spatial and temporal resolution (0.5–6 mm and seconds to minutes, respectively); dependence on a special environment	Frick et al. (2015), Spindelegger et al. (2009), Moses (2011)
GESIs	5-HT dynamics are transformed to a fluorescent readout from genetically encoded probes	A fluorescence signal readout is induced by 5-HT binding to sensors	High spatiotemporal resolution; high specificity and sensitivity	Reliance on genetically encoded probes; invasiveness of imaging device implantation; novelty of technology and lack of validation for long-term use	Unger et al. (2020), Dong et al. (2021), Wan et al. (2021)

Abbreviations: 5-HT, 5-hydroxytryptamine; fMRI, functional magnetic resonance imaging; FSCV, fast-scan cyclic voltammetry; GESIs, genetically encoded serotonin indicators; PET, positron emission tomography.

flexed vectors, which means scientists can measure the dynamics of serotonin in defined types of cells.

For imaging modality, GESIs are validated by wide-field microscopy, fiber photometry, and two-photon microscopy. In the future, GESIs can be combined with newly developed modalities, such as microprisms to image across midline fissures (Andermann et al., 2013; Chia & Levene, 2009) and gradient index (GRIN) lenses to image deep brain structures (Ghosh et al., 2011; Yu et al., 2017), which are beginning to afford optical access to historically inaccessible brain regions. The various imaging modalities provide choice space in terms of behavioral study requirements and conditions. Imaging serotonin signals by GESIs in the brain enables recording activity patterns in local circuits. The development of GESIs is poised to open a new chapter in physiological experiments and pharmacological approaches in the development of novel therapies.

In contrast to such advantages, there are several limitations of GESIs that currently preclude their widespread application to study neurotransmitter signaling. First, when compared to sensors for calcium, glutamate, and dopamine, which are well developed, the performance of serotonin sensors within cells remains to be optimized. In particular, all GESIs expressed in vivo lost a significant proportion of their fluorescence dynamic range. In the case of iSeroSnFR, its peak fluorescence change in vivo is measured at only approximately

12% $\Delta F/F_0$ during the sleep–wake cycle, a tiny fraction of its fluorescence response in cultured (HEK) cells (documented at 1700% (Unger et al., 2020); Table 2). As with $GRAB_{5\text{-}HT1.0}$, the peak fluorescence change following injection of MDMA is detected at approximately 55% $\Delta F/F_0$ in vivo, compared to 250% recorded in cultured HEK cells. This is in contrast to the glutamate sensor SF-iGluSnFR, which is highly sensitive and is a robust fluorescent sensor of glutamate transients in dendritic spines and axonal boutons (Marvin et al., 2018).

The second limitation of GESIs is that, while they are much more compact in design and can be packaged as cDNA expression constructs for viral vector delivery (e.g., AAVs), their introduction into the brain still requires physical manipulation of brain tissue. Relatedly, viral toxicity in host tissue and cell types are also possible confounds. Furthermore, once introduced into cells, GESIs maintained episomally are cleared in cells or diluted as host cells divide, leading to changes in the expression levels of such reporters within targeted cells.

The third limitation is that over-expression of genetically encoded sensors, themselves exogenous proteins, may unduly influence intracellular homeostasis, lead to cell membrane crowding or result in cytotoxicity. Indeed, it is recognized that sensors based on GPCR binding domains such as psychLights and other GRAB biosensors could potentially interrupt downstream signaling pathways for endogenous GPCRs. Furthermore, for endogenous receptors, the availability of ligands can be reduced by ligand buffering caused by sensor over-expression, especially for sensors such as $GRAB_{5\text{-}HT1.0}$, with high serotonin affinity and slow off kinetics.

The fourth limitation concerns the imaging process. The major in vivo imaging methods for GESIs are optical fibers and two-photon microscopy. For optical fibers, animals need to be implanted with an optical fiber, which is invasive for brain tissue. For imaging devices such as two-photon microscopes, scientists need to shave the bone to provide the imaging window for head-fixed imaging devices.

Thus, while GESIs are the most recent addition to the toolbox for neuroscientists, they still require extensive validation and characterization, specifically for longitudinal studies of the brain in the context of awake behaviors.

4 | CONCLUSIONS AND PERSPECTIVES

In the decades since the discovery of serotonin in the 1930s–1940s, various technologies have been developed to lift the veil of mystery that has shrouded this molecule (Figure 2). The major application of these tools is to investigate causal relationships between serotonin neurotransmission and behavior. Understanding the serotonin regulation mechanism is also helpful for understanding serotonergic dysfunction and developing effective treatments. To understand the serotonin dynamics underlying complex behavior regulation, it is often necessary to trace neurons over behaviorally relevant time scales, for example, longer than several hours for sleep–wake cycles. Many serotonin-related psychiatric diseases, such as depression and autism spectrum disorder, develop progressively over long periods of time and are influenced by diverse factors, leading to a spectrum of clinical presentations. In order to understand the role of 5-HT in disease development, 5-HT activity needs to be traced in vivo over an extended period, which requires a detection method suitable for long-term measurements. Among the methods we reviewed, microdialysis performs better than the other methods with regard to long-term and recurrent detection. For example, commercially available microdialysis systems were tested for up to 18 months in mice. As for novel GESIs, these tools have not yet been validated for long-term use in vivo. It must be admitted that there is still a massive gap in neural activity and behaviors between general model animals (such as mice and rats in neuroscience) and human beings. Circuits and functions involved in disease development and behavior studies are different between different organisms. Humans are the most appropriate model for serotonergic function research, so detection methods must be non-invasive for in vivo studies. From this perspective, BOLD fMRI and PET are more attractive options than other methods although fMRI and PET still cannot be considered as fully non-invasive. Moreover, the spatiotemporal resolution of tomography still needs to be improved in future applications. Emerging GESI technology provides superior spatiotemporal resolution and sensitivity. This set of optical sensors enables the visualization and tracing of neural activity in real time. Neural activity is complex and usually regulated by various neurochemicals. Multiplexed imaging of biosensors for detecting different neurochemicals is a major trend in neuroscience research that requires expanding the color palette and biochemical diversity of serotonin sensors. For instance, redshifted probes allow deeper and more efficient imaging in vivo, which has further advantages for deep tissue imaging because of lower scattering and absorption.

There is no single tool that can fulfill detection demands in all dimensions. While this review is focused on methods for serotonin detection in vivo, it should be noted that a holistic understanding of serotonin function and serotonergic systems requires comprehensive approaches involving genomics, transcriptomics, proteomics, neurochemistry, electrophysiology, and behavioral studies. End users should select tools depending on the experimental design and requirements. Close collaboration between end users and developers will also be needed to reconcile practical and technical barriers to tools and their widespread use across research, clinical, and public health settings. In particular, new methods taking into account the complexity and longitudinal impact of serotonin signaling in awake and behaving animals across weeks and months would be highly valuable. We observe that, historically, detection methods follow a pattern of adoption, widespread use that reaches a plateau, followed by decline, particularly for techniques such as microdialysis (Figure 2). We are still only beginning to gain insights into the mechanism of serotonergic activity with the help of those detection methods. More advanced technologies may provide more accurate serotonergic activity and even distinguish the activity of individual neurons. Until then, researchers will continue to analyze, measure, develop, and innovate in the quest to understand the remarkable role of serotonin in the brain.

AUTHOR CONTRIBUTIONS

Kiryl D. Piatkevich: Conceptualization, supervision, and funding acquisition. Shuchang Zhao: Writing—original draft preparation. Kiryl D. Piatkevich and Shuchang Zhao: Writing—review and editing, visualization. All authors contributed to the article and approved the submitted version.

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DATA AVAILABILITY STATEMENT

The data are available from the corresponding authors of the paper upon request.

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