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A Fluorescence Displacement Assay for Antidepressant Drug Discovery Based on Ligand-Conjugated Quantum Dots

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Supporting Information

ABSTRACT: The serotonin (5-hydroxytryptamine, 5-HT) transporter (SERT) protein plays a central role in terminating 5-HT neurotransmission and is the most important therapeutic target for the treatment of major depression and anxiety disorders. We report an innovative, versatile, and target-selective quantum dot (QD) labeling approach for SERT in single *Xenopus* oocytes that can be adopted as a drug-screening platform. Our labeling approach employs a custom-made, QD-tagged indoleamine derivative ligand, IDT318, that is structurally similar to 5-HT and accesses the primary binding site with enhanced human SERT selectivity. Incubating QD-labeled oocytes with paroxetine (Paxil), a high-affinity SERT-specific inhibitor, showed a concentration- and time-dependent decrease in QD fluorescence, demonstrating the utility of our approach for the identification of SERT modulators. Furthermore, with the development of ligands aimed at other pharmacologically relevant targets, our approach may potentially form the basis for a multitarget drug discovery platform.

Major depression occurs in 2−5% of the U.S. population and is the most common mental illness in modern society.[1] Depression is not only devastating but also presents a financial burden, costing the U.S. an estimated 100 billion dollars annually.[1b] Selective serotonin reuptake inhibitors (SSRIs) that block the serotonin transporter (SERT) at brain synapses are by far the most frequently prescribed drugs for the management of depression.[1a,2] A well-known major drawback of current SSRIs is their slow onset of antidepressant activity, requiring 3−6 weeks of administration to produce a significant therapeutic benefit.[3]

To develop faster-acting antidepressants, a multitarget strategy, wherein antagonists are designed for several pharmacologically relevant targets, has been proposed.[3] Several studies have indicated that dual-acting antidepressants such as Desvenlafaxine,[4] a serotonin and norepinephrine reuptake inhibitor (SNRI), and SB-649915-B,[5] a 5-HT$_{1A/B}$ receptor antagonist and SSRI, may provide a faster onset of antidepressant action. Another emerging area in antidepressant drug discovery exploits allosteric antagonists.[6] In this approach, drug candidates can be engineered to act at a site of the transporter distinct from the high-affinity primary binding site, consequently mediating conformational changes of a substrate binding pocket and attenuating neurotransmitter uptake. No crystal structure of any neurotransmitter transporter is presently available, which makes it difficult to validate the allosteric antagonism. Several high-affinity SSRIs have been previously proposed as allosteric modulators for SERT, including paroxetine, (Paxil), a high-affinity SERT-specific inhibitor and FDA-approved SSRI.[7]

Recently, several new multitarget antagonists and allosteric modulators have shown improved efficacy and success in clinical trials. However, progress in next-generation antidepressant drug discovery has been largely delayed by the lack of appropriate screening platforms.[3] At present, methods used to investigate transporter binding/activity rely on conventional biochemical methods such as in vitro phosphorylation assays, electrophysiology,[8] or radiolabeled substrate uptake assays.[8] These methods are labor-intensive and time-consuming, and the radiolabeling method requires isotope use.

Alternatively, fluorescent probes can be used for target-selective drug screening. However, when common fluorophores are used, the two major limiting features are photostability and sensitivity. In recent years, the development of quantum dots (QDs) has achieved promising results that overcome the disadvantages associated with conventional biolabeling fluorophores.[9] We previously demonstrated the use of ligand-conjugated QDs for visualization of SERT, the GABA$_C$ receptor, and most recently the dopamine transporter.[10] In this report, we advance the ligand-conjugated QD labeling approach as an antidepressant drug-screening platform in single, living oocytes.

Figure 1 illustrates two modes by which ligand-conjugated QD displacement can occur. The first mode involves preventing ligand reassociation with the primary (orthosteric) binding site (left), and the second mode operates through an allosteric mechanism that shifts the primary binding site conformation, causing ligand dissociation (right).

The structure of the IDT318 ligand used in this study is depicted in Figure 2A. The ligand design was based on comprehensive screening of trypamine derivatives,[11] and the synthesis details have been described previously.[12] As indicated, the IDT318 ligand is composed of

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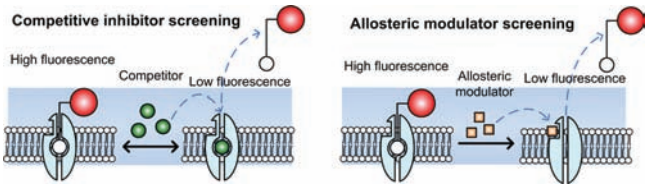

Figure 1. Fluorescence displacement assay based on ligand-conjugated QDs for antidepressant drug discovery. Target proteins (transporters or receptors) bind to the QD-tagged ligands, forming complexes that increase the fluorescent signal along the membrane. Upon exposure to a potential drug that competes with the binding (left) or induces a conformational change in the binding site (right), the QD-tagged ligands are displaced, resulting in a decrease in fluorescence intensity. The blue-shaded area indicates the imaging focal plane while processing the assay.

four components. 5-Methoxy-3-(1,2,5,6-tetrahydro-4-pyridinyl)-1*H*-indole (RU24969), which retains the tryptamine moiety for a putative common 5-HT binding site and features enhanced selectivity for human serotonin transporter (hSERT),[11] is readily adapted as a tethered ligand for hSERT binding[13] (component I). The alkyl spacer serves to enhance the ligand binding through the interaction of the hydrophobic residues in the transmembrane domains of membrane channels or transporters [component II; also see Figure S1 in the Supporting Information (SI)]. The polyethylene glycol (PEG) chain is used to increase the water solubility of the ligand and decrease steric hindrance from the bulky QD (component III). The biotin group (component IV) allows for specific binding to the streptavidin-conjugated QD (SA−QD). In addition, only surface PEGylated SA−QDs were used because of their ultralow nonspecific binding property.[14]

To visualize the hSERT distribution in our oocyte model, a two-step labeling approach was implemented in which an hSERT-expressing oocyte was incubated with IDT318 and then with SA−QDs. As shown in Figure 2B (column 1), the QD fluorescence formed a sharp halo correlating to the membrane of the hSERT-expressing oocyte, whereas incubation with nonexpressing control oocyte showed no sign of labeling (column 2). Ligand binding specificity was demonstrated using a QD-based preincubation affinity assay where the hSERT-expressing oocyte was preincubated with paroxetine prior to the two-step QD labeling. As can be seen in column 3, paroxetine effectively blocked the QD labeling, demonstrating the binding specificity of IDT318 to hSERT. In comparison, preincubation of an hSERT-expressing oocyte with 0.1 mM 5-HT prior to the two-step QD labeling showed only reduced QD fluorescence intensity (column 4). This reduced QD labeling could be the result of incomplete saturation of hSERT binding with 5-HT; however, this is unlikely since the 5-HT concentration was 120 times greater than the reported K_i value against hSERT.[11] A more likely explanation is the reversible binding mode, in which IDT318 competes with 5-HT for the primary binding site. This rationale is also consistent with our previous finding that tryptamine analogues, including RU24969, share a common substrate binding site at hSERT.[11] Importantly, the influence of IDT318 on the hSERT activity was further characterized using an oocyte electrophysiological assay (Figure S1), indicating the role of IDT318 as an hSERT antagonist.

The potential utility of our labeling model for SSRI screening was explored utilizing paroxetine. For the drug candidate to displace the fluorophore-tagged ligand rapidly at a reasonable drug concentration, a ligand with an affinity in the micromolar range is required.[15] As indicated in Figure S2, IDT318 shows the

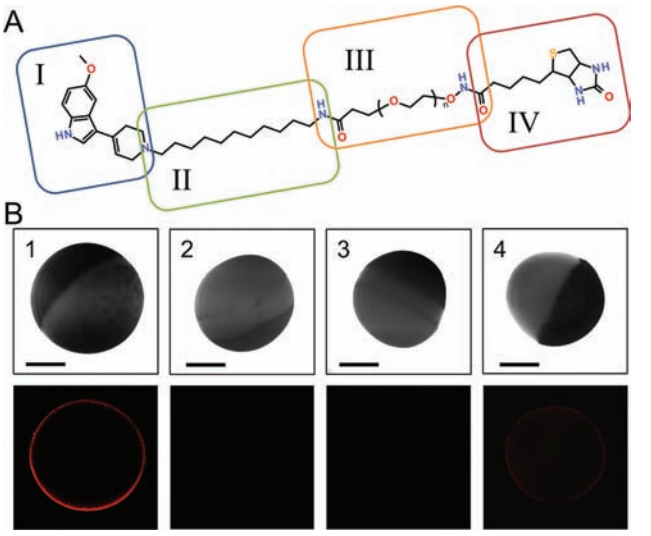

Figure 2. Target-selective QD-SERT labeling via IDT318. (A) Structure of the IDT318 ligand used in this study (see the text for details of each component). (B) Column 1: Incubation of hSERT oocyte with 1 μM IDT318 ligand prior to 2.5 nM SA−QD treatment. The observed QD fluorescence forms a sharp halo correlating to the membrane of hSERT expressing oocyte. Column 2: Control experiment where the buffer-injected oocyte was treated under the same conditions as in column 1. Column 3: hSERT-expressing oocyte was incubated with paroxetine (1 μM) and subsequently exposed to the ligand/paroxetine mixture (1 μM/1 μM) prior to 2.5 nM SA−QD treatment. Column 4: hSERT-expressing oocyte was preincubated with 5-HT (1 mM) and subsequently exposed to the ligand/5-HT mixture (1 μM/1 mM) prior to 2.5 nM SA−QD treatment. The halo was dimmer but not completely blocked, indicating a competitive binding mechanism. Upper panels are differential interference contrast (DIC) images, and the lower panels are fluorescent images. Scale bars = 0.5 mm. The results are representative micrographs from at least three independent experiments.

desired micromolar affinity. In our displacement assay, a 30 min time-course imaging at 1 min intervals was carried out immediately after paroxetine incubation (see the SI for details). The QD fluorescence intensity was measured and normalized as F_t/F_0, where F_0 is the initial fluorescence signal and the F_t is the fluorescence signal at time t. Representative time-lapse fluorescent images and fluorescence intensity traces are shown in panels A and B of Figure 3, respectively. Time- and concentration-dependent fluorescence intensity reduction is apparent in the presence of paroxetine. In contrast, when 5-HT was used as the displacing drug, the dramatic QD fluorescence reduction seen with paroxetine treatment vanished (Figure S4). It should be noted that the reduction in the fluorescence intensity after 30 min of buffer incubation was less than 10% (Figure 3B), indicating that the effect of QD quenching and spontaneous ligand dissociation minimally contributes to the results. Furthermore, the plot of $\log(F_t/F_0)$ as a function of time was linear over the first 10 min, indicating first-order dissociation kinetics (Figure 3C). Analysis of the 10 μM paroxetine displacement trace yielded an apparent dissociation rate constant (k_{app}) of $(5.0 \pm 0.4) \times 10^{-4}\ s^{-1}$. Doubling the paroxetine concentration resulted in a nearly proportional increase in k_{app} to $(1.08 \pm 0.05) \times 10^{-3}\ s^{-1}$ (see the SI for fitting details). Hence, this displacement platform exhibits the sensitivity necessary for SSRI screening. Importantly, the dissociation kinetics shown in Figure 3C indicate that the time-series displacement can be performed in less than 10 min. From a

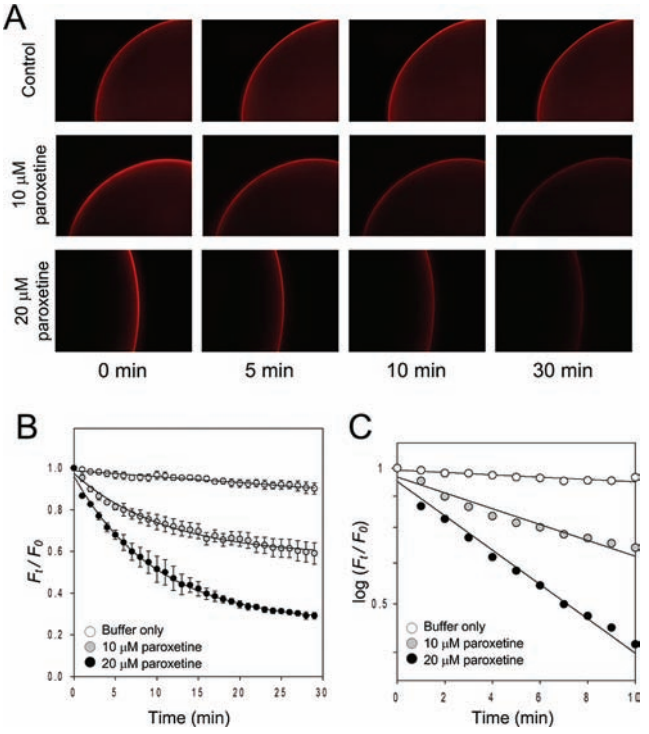

Figure 3. Displacement analysis of QD-ligand-labeled hSERT with paroxetine. The representative time-lapse QD fluorescence images (A), time-dependent relative fluorescence intensity plot (B), and kinetic analysis (C) show the effect of paroxetine on ligand−SERT displacement in the presence of PBS buffer (control) and 10 and 20 μM paroxetine. The solid lines in (B) reflect best fits (see the SI for details). The log-scale plot of the displacement curves (C) was fit to a linear function, indicating first-order dissociation kinetics. Each data point in (B) and (C) represents the mean of three independent measurements. Data points in (B) are given as mean ± standard deviation. The results in (A) are representative micrographs from at least three independent experiments.

technological perspective, the throughput of our QD-based displacement assays can be increased more than 100-fold if used with a commercially available automated multiwell-plate high-throughput screening imaging system.

The ability of paroxetine to displace IDT318 at SERT is most likely to be a noncompetitive, allosteric mode of interaction of the antidepressant with the transporter, as opposed to a simple competition for an orthosteric binding site.[7,16] Recently, it was shown that mutations at the major antidepressant binding site of SERT do not impact paroxetine, also suggesting a noncompetitive mode of interaction.[13] Additionally, noncompetitive dissociation of ligands from binding sites is expected to follow first-order dissociation kinetics, as in the case of *S*-citalopram,[17] an SSRI frequently proposed to interact with SERT via an allosteric mechanism.

In conclusion, we have demostrated a fluorescence displacement assay for antidepressant drug discovery based on ligand-conjugated QDs. Furthermore, our method is the first target-selective drug discovery platform that utilizes fluorescent QDs. This system may aid in mapping allosteric mechanisms of SERT modulation and potentially form the basis for a multitarget drug discovery platform employing ligand-conjugated QDs that selectively bind to other pharmacologically relevant proteins, such as dopamine transporter[10c] and norepinephrine transporter. Ultimately, this platform may provide more insight into the effects of different structural features on the binding kinetics of any ligand−protein interaction and therefore serve as a generalized approach for the development of drugs beyond antidepressants.

■ ASSOCIATED CONTENT

Supporting Information. Experimental section and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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