Labeling of neuronal receptors and transporters with quantum dots

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The ability to efficiently visualize protein targets in cells is a fundamental goal in biological research. Recently, quantum dots (QDots) have emerged as a powerful class of fluorescent probes for labeling membrane proteins in living cells because of breakthrough advances in QDot surface chemistry and biofunctionalization strategies. This review discusses the increasing use of QDots for fluorescence imaging of neuronal receptors and transporters. The readers are briefly introduced to QDot structure, photophysical properties, and common synthetic routes toward the generation of water-soluble QDots. The following section highlights several reports of QDot application that seek to unravel molecular aspects of neuronal receptor and transporter regulation and trafficking. This article is closed with a prospectus of the future of derivatized QDots in neurobiological and pharmacological research. © 2012 Wiley Periodicals, Inc.

INTRODUCTION

Since the pioneering neuroanatomical work of Santiago Ramón y Cajal in the early 20th century, the need to visualize dynamic communication within neuronal networks has been a challenge for modern neuroscience.[1] Synaptic transmission, and hence central nervous system excitability, are modulated in a tightly regulated manner by a myriad of neuronal membrane receptors and transporters that rely on specific ligand binding and transport and the initiation of intracellular signaling cascades. Despite extensive biochemical and genetic analyses, mechanisms that regulate synaptic receptor and transporter activity, trafficking, and localization continue to challenge neuroscientists.[2,3] These considerations have driven our desire to combine recently developed receptor and transporter labeling methods with advanced fluorescence imaging tools to dissect mechanisms of synaptic protein trafficking and signaling at the single-molecule level.

Fluorescent labeling techniques commonly used to interrogate cellular processes can be classified into two broad categories: (1) the construction and expression of fluorescent fusion proteins such as green fluorescent protein (GFP)[4,5] and (2) the chemical methods of fluorescent labeling.[6] With the rapid evolution of fluorescent protein technology, GFP-based labeling methods have quickly found widespread use as the proteins produced bypass many

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time-consuming probe preparation steps and assure that labeling is restricted to the desired proteins. Unfortunately, there are certain limitations associated with the GFP fusion approach: (1) incompatibility of GFP construct transfection with endogenous expression systems; (2) failure of GFP-tagged proteins to localize properly; and (3) differences in the activity and signaling of GFP-tagged proteins compared to their wild-type counterparts. As a result, alternative fluorescence labeling strategies have emerged for the visualization of cellular proteins, particularly those systems that are incompatible with the GFP-based methodology. However, as chemical labeling techniques typically utilize conventional fluorescent dyes, live-cell imaging is often hampered by the low photostability and brightness, narrow Stokes shift, and relatively broad emission spectra of these labels. To address these shortcomings, researchers have developed fluorescent nanomaterials with significantly improved optical properties for biological imaging.

Semiconductor nanocrystals, also known as quantum dots (QDots), are one such class of fluorescent nanomaterials that overcome instability issues associated with conventional fluorescent dyes and support versatile protein labeling via their surface functionalization with various targeting probes.[7–9] More importantly, QDots are much better suited to advanced fluorescence imaging approaches that pursue live-cell, multicolor detection, *in vivo* animal imaging,[10–12] and single-molecule tracking.[13,14] Here, an overview of recent advances in QDot synthesis and the conjugation of biological probes to the QDot surface is provided. In addition, several reports of QDot application that seek to unravel molecular aspects of neuronal receptor and transporter regulation and trafficking are highlighted. This article is closed with a prospectus of the future of derivatized QDots in neurobiological and pharmacological research.

WATER-SOLUBLE QDots

What Is a QDot?

QDots are nanometer-sized crystals that are composed of several hundreds to thousands of atoms of periodic group II/VI (CdSe, CdTe, CdS), IV/VI (PbS, PbSe), III/V (InP), or ternary (InGaP) semiconductor materials, with CdSe being one of the most commonly encountered materials.[7,15–19] Typically, the first step of QDot preparation involves high-temperature pyrolysis of organometallic precursors in organic solvents, such as trioctylphosphine (TOP), trioctylphosphine oxide (TOPO), and hexadecylamine[17,18] As a result,

the as-synthesized QDot is composed of a CdSe core capped with a layer of hydrophobic ligands. These 'bare', water-insoluble QDots cannot be immediately used in an aqueous, biological environment; moreover, these QDots are susceptible to degradation/aggregation and suffer from relatively low fluorescence quantum yields as a result of the nonradiative recombination processes occurring at the core surface. To eliminate these problems, the CdSe core is commonly passivated with a shell of a wider bandgap inorganic material (ZnS) to increase its stability and optimize optical properties.[16,18] Thus, the term 'quantum dot' usually refers to a CdSe/ZnS core/shell structure. Figure 1(a) shows an atomic number-contrast scanning transmission electron microscopy (Z-STEM) image of a single CdSe/CdS/ZnS core/shell QDot commercially available from Life Technologies (Grand Island, NY, USA).[7]

QDot Properties

The properly passivated CdSe core gives rise to a unique combination of photophysical properties of QDots. QDots are characterized by remarkable brightness, which is a product of their high molar extinction coefficients ($100,000–1,000,000$ $M^{-1}cm^{-1}$) and near-unity quantum yields.[7,15,21] In addition, their broad absorption spectra and narrow Gaussian emission spectra considerably simplify multicolor experiments, allowing visualization of several fluorophores using a single, inexpensive excitation source with minimal cross talk (Figure 1(b)).[22–26] The dependence of the emission wavelength on the size of the QDot core is a key property of QDots (Figure 1(c)), providing for easy diversification of probe identity through relatively simple alterations in chemical synthesis. The inorganic nature of QDots and passivation of the QDot core render QDots extremely resistant to photobleaching.[17,23,27] This optical stability is an important advantage over organic dyes and fluorescent proteins; superior QDot photostability allows for long-term visualization of dynamic biological processes (Figure 1(d)). An interesting and pivotal feature inherent in the emission spectra of single QDots is the alternation of 'dark' (off) and 'bright' (on) states, termed 'blinking' (Figure 1(e)).[28–32] Although such fluorescence intermittency can be used as a criterion to assure the observation of a single QDot, this also remains problematic when QDot tracking experiments are performed. Finally, another key advantage of QDots over organic dyes and fluorescent proteins is the versatility of QDot surface modification, a feature that is discussed in greater detail below.
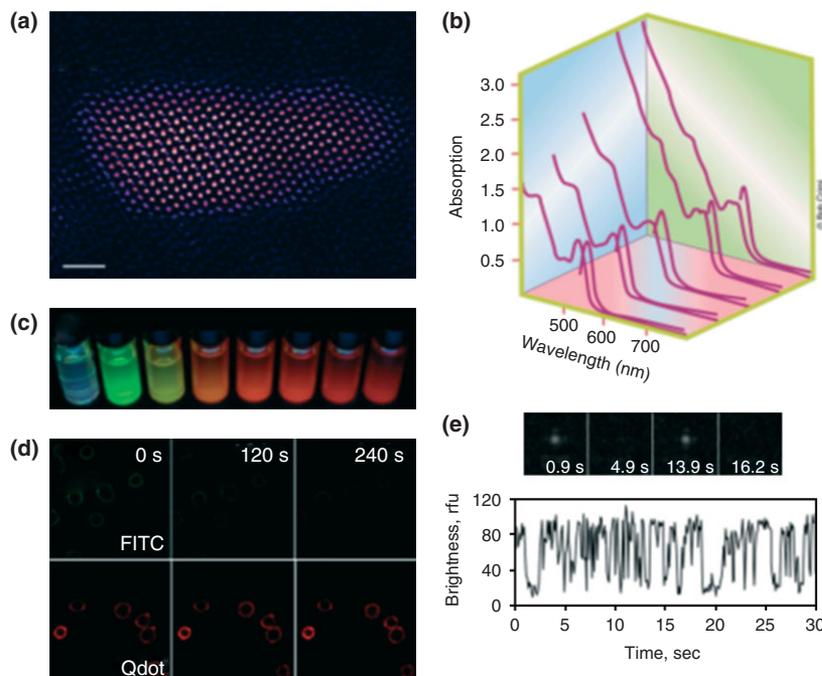
FIGURE 1 | Photophysical properties of quantum dots. (a) Aberration-corrected Z-STEM image of a commercial core/shell QD655 (Life Technologies). It can be seen that the core/shell QDot is actually an elongated, bullet-shaped 3D object rather than a sphere. (Reprinted with permission from Ref 7. Copyright 2007 Elsevier B.V.) (b) Absorption and emission spectra of a series of CdSe nanocrystals. The size of the QDot core determines the absorption and emission spectra of QDots. (Reprinted with permission from Ref 14. Copyright 2001 Nature Publishing Group) (c) A series of UV-illuminated CdSe nanocrystals ranging in size from ~2 to ~6 nm. (d) Time-lapse image series of FITC- and QD-labeled HEK-293 cells. In contrast to traditional fluorophores, QDots are characterized by excellent photostability, which enables long-term monitoring of biological processes. (Reprinted with permission from Ref 20. Copyright 2011 American Chemical Society) (e) Fluorescence intermittency or 'blinking' in the emission spectrum of a single QDot. This QDot property can be used as a criterion to distinguish single nanocrystals from aggregates.

Solubilization

As mentioned above, as-synthesized core/shell QDots are capped with hydrophobic organic ligands and are thus incompatible with an aqueous biological environment. To render QDots biocompatible, one must first make them soluble in water while preserving their photophysical properties. Several strategies used to achieve this are (1) ligand exchange, (2) polymer encapsulation, and (3) silica shell encapsulation (Figure 2). Before discussing these approaches, it should be noted that any solubilization route must lead to a QDot that is stable under physiologically relevant conditions, that displays minimal nonspecific interactions, and possesses functional surface elements necessary for the introduction of target specificity. In the ligand-exchange approach, bifunctional ligands, which contain a point of attachment to the QDot surface on one end and a hydrophilic moiety on the other, displace native organic ligands (Figure 2(a)).[33] In polymer encapsulation, native organic ligands are retained, and instead, tightly associate with the inner aliphatic chains of an added amphiphilic polymer shell.[11,34,35] The exterior hydrophilic backbone of the

polymer then aids in aqueous dispersion (Figure 2(b)). With silica encapsulation, as-synthesized QDots are encapsulated within an inert silica shell (Figure 2(c)).[36–38] The silica encapsulation strategy is not yet in wide use because of the tendency of silica-coated QDots to agglomerate during synthesis as well as lack of control over the growth of silica spheres around QDots. Depending on the experimental requirements, each of the above solubilization strategies may be the optimal method. Amphiphilic polymer encapsulation has thus far been the preferred strategy because polymer-coated QDots retain native surface-capping ligands and do not exhibit significant decrease in performance, e.g., quantum yield. In addition, polymer-coated QDots are characterized by superior colloidal stability in physiologically relevant conditions. However, the added polymer shell significantly increases the hydrodynamic diameter of QDots, limiting their use in size-sensitive applications. With ligand exchange, it is possible to maintain QDot size below the typical hydrodynamic diameter values of 30–40 nm after polymer encapsulation while preserving the original photophysical properties. It
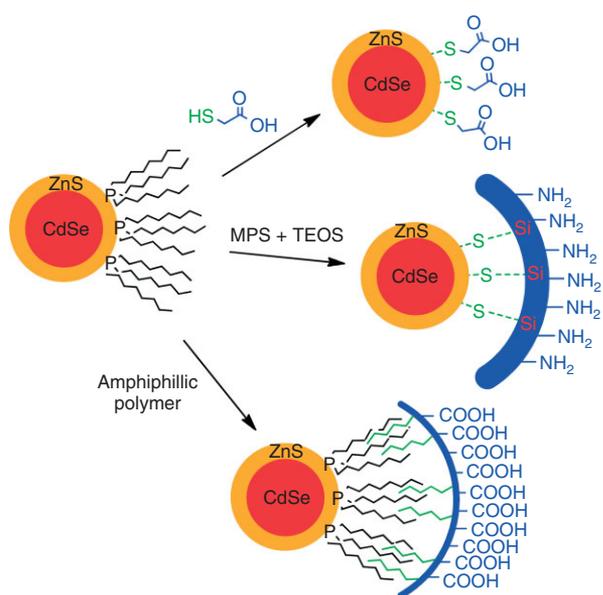
FIGURE 2 | Quantum dot solubilization approaches. The as-synthesized QDots can be rendered soluble in water via ligand exchange (top), wherein native hydrophobic surfactants are replaced with bifunctional hydrophobic capping ligands; via encapsulation in an insert silica shell (middle); or via amphiphilic polymer encapsulation (bottom), wherein native surfactants are retained and integrated into the polymer shell.

is true that in some cases, the exchange of native surface-capping ligands leads to significant decrease of quantum yield and shelf life; however, the use of compact, polydentate ligands (dihydrolipoic acid) is a potential solution to these problems.[39,40]

To summarize, the goal of solubilization is to modify the QDot surface in such a way that the resulting QDots are water soluble and stable at physiologically relevant pH and ionic strength ranges. One must carefully ensure that such postpreparative surface modification is achieved with modified QDots that retain their original physical and optical properties. Finally, water-soluble QDots must contain functional reactivity handles to allow subsequent conjugation of biospecific molecules to the QDot surface.

CONJUGATION OF BIOPROBES TO QDots

Once QDots have been made water soluble, the next step is to introduce biological specificity for a specific target receptor or transporter. QDots present a very attractive photoluminescent scaffold with a high surface-area-to-volume ratio, enabling conjugation of multiple copies of various reactivity

handles and biospecific recognition elements to the QDot surface. In addition to a consideration of the role of bioprobe-binding valency to QDots, which is described in detail later, a prerequisite for success in QDot labeling is the choice of an appropriate bioprobe. Bioprobes that have been introduced in QDot conjugation include small organic ligands, peptides, adhesive proteins (i.e., streptavidin), and antibodies. Each of these probes presents particular advantages and disadvantages (see Table 1 for comparison). QDot–antibody nanoconjugates have thus far been the preferred method of surface protein detection as they provide excellent specificity, and besides commercial antibody conjugation kits are readily available. However, QDot–antibody conjugates typically possess a large hydrodynamic diameter, which may complicate the use of such probes in crowded cellular locations. In addition, the long-term stability of the antibody–protein interaction may be a concern as antibody probes are prone to proteolytic digestion and degradation. The potential for cross-linking of proteins, which serves as an activation signal for some receptors, is another problem, though the use of Fab fragments can overcome this issue. Peptide-based QDots and antibody-conjugated QDots share similar features in that probes are also readily available and they are both prone to proteolytic digestion. A significant advantage with peptide-conjugated QDots is that several neuropeptide receptors still lack small-molecule ligands and specific antibodies for targeting. Biological recognition can also be incorporated in the QDot architecture through the use of biologically active small-molecule ligands. These ligand-conjugated QDots rely on ligand–receptor interactions to provide specificity. Additionally, a ligand-based targeting approach can be designed to elicit either an agonistic or antagonistic physiological response upon QDot probe binding to the target protein. While the ligand-conjugated Qdots may potentially address the concerns regarding poor specificity associated with the peptide-based QDot conjugates, fairly sophisticated organic chemistry is required to synthesize small-molecule ligand probes for conjugation to the QDot surface. In addition, rigorous ligand characterization and purification steps must be involved. Each class of bioprobes that can be conjugated to QDots is associated with distinct advantages and disadvantages, and bioprobe selection will ultimately be dependent on experimental conditions and goals.

To take advantage of the unique photophysical properties of QDots for live-cell real-time imaging of surface proteins, the choice of conjugation methodology should be considered as important

TABLE 1 | Advantages and Weaknesses of Major Classes of Functional Probes that Can Be Conjugated to QDots for Biological Labeling and Imaging

Targeting Probe	Advantages	Disadvantages
Organic ligand	• Uses known small-molecule drugs/agonists/inhibitors; a much more diversified base of ligand candidates compared to peptides/antibodies. • Readily applied to binding/affinity assays and structure–function relationship studies. • Potential in pharmacological or physiological applications.	• Lack of commercially available sources. • Fairly sophisticated organic chemistry is required for ligand synthesis and purification. • Requires rigorous spectroscopic analyses. • Requires additional pharmacological analyses. • Potential issues with multivalency of the final conjugates.
Peptide	• Readily available custom peptide synthesis services and well-established solid-phase synthesizers on the market. • Applicable for intracellular targets when combined with cell penetrating peptides. • Good biocomparability. • Low cellular toxicity in general compared to organic ligand. • Particularly useful for certain neuropeptide receptors.	• Fewer reported peptides applicable for membrane protein targeting. • Relatively short half-life in general compared with organic ligands; prone to degradation mechanisms and proteolytic digestion. • May require additional pharmacological analyses. • Relatively poor receptor specificity compared to the monoclonal antibody.
Antibody	• Rich commercially accessible and feasible resources. • Routinely used in biochemical and cell-based assays; gold standard in protein detection/labeling. • Excellent biocompatibility; low cellular toxicity concern in most cases.	• Bulky, significantly increase the hydrodiameter after conjugation. • Concerned with batch-to-batch variability, particularly with the use of polyclonal antibodies. • Prone to degradation mechanisms and proteolytic digestion.

as the choice of the bioprobe. Several methodologies have been devised to functionalize QDots with target-specific bioprobes and can be grouped into two categories: direct and indirect conjugation. These QDot conjugation approaches are discussed below along with specific examples of their use in targeting neuronal receptors and transporters.

Direct Conjugation Approach

In the direct conjugation approach, a reactive group at the QDot surface (–COOH,–NH$_2$,–SH) is conjugated to a biospecific molecule that has a compatible reactivity handle. This is achieved through the use of heterobifunctional cross-linker molecules (Figure 3), such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). In EDC coupling, QDot surface carboxyl groups (–COOH) are first reacted with EDC reagent to form an unstable, amine-reactive O-acylisourea intermediate that readily reacts with an amino-terminated (–NH2) biomolecule to form an amide bond. Typically, the unstable amine-reactive intermediate is stabilized using *N*-hydroxysuccinimide; the NHS-stabilized intermediate then quickly reacts with an amino-terminated biomolecule to form an amide bond. In

SMCC coupling, SMCC or its water-soluble analog sulfo-SMCC reacts with QDot surface amino groups to yield maleimide-activated QDots. Then the maleimide-activated QDots are reacted with a biomolecule that contains a sulfhydryl (–SH) group to produce QDot conjugates. Similar to SMCC coupling, SPDP coupling allows conjugation of amino-terminated QDots to sulfhydryl-containing biomolecules, or vice versa. Amino-terminated QDots first react with SPDP to give 2-pyridilthio-terminated QDots, with the 2-pyridil groups readily displaced by sulfhydryls under the neutral pH conditions. The EDC/NHS methodology is typically used to covalently conjugate small molecules, polyethylene glycol (PEG) chains, or high-affinity proteins (avidin) to the surface of water-soluble QDots, whereas the SMCC/SPDP methodology is commonly used to conjugate antibodies or antigen-binding fragments (Fab) to QDots. To do so, the hinge disulfide bonds of an antibody or a Fab fragment must first be reduced with dithiothreitol to yield free sulfhydryl groups.[41] Another way to confer specificity to the QDot surface is to combine solubilization and subsequent functionalization steps into a one-step ligand-exchange procedure.[8] Such a strategy involves the exchange of native capping ligands with heterobifunctional, amphiphilic ligands that contain a chemical group, which may be directly conjugated to the bare nanocrystal (e.g.,–SH) as well
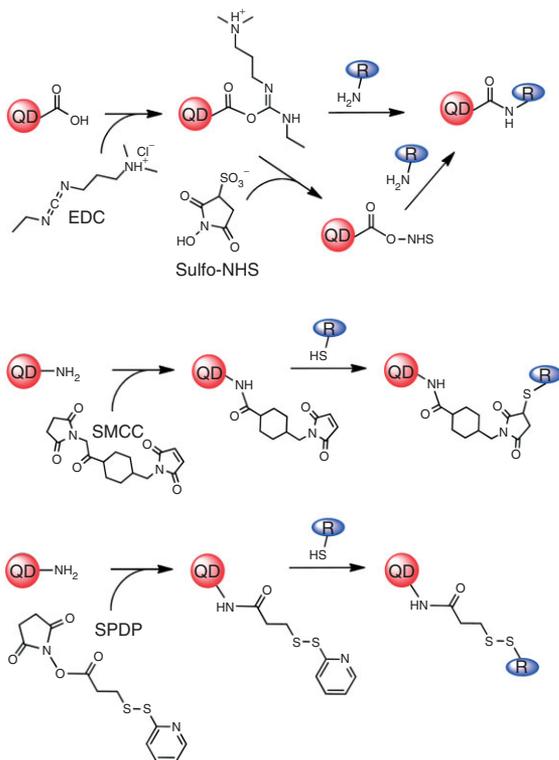
FIGURE 3 | Direct conjugation methodology. Schematic diagram of the chemical reactions that occur during covalent coupling of bioprobes to the QDot surface by using EDC/NHS (top), SMCC (middle), and SPDP (bottom) reagents.

as a functional element at the opposite ligand end that facilitates binding to a neuronal receptor or transporter.[42]

Indirect Conjugation Approach

In the indirect conjugation approach, a biospecific molecule is not directly conjugated to the surface of the QDot. Instead, this approach takes advantage of high-affinity noncovalent interactions between high-affinity binding partners, such as avidin family proteins that bind biotin and biotin-tagged molecules. Although other partner molecules exist for such use (e.g., maltose and maltose-binding protein), avidin–biotin noncovalent assembly is the most popular approach to achieve stable, target-specific QDot labeling and thus is discussed in more detail below.

The use of avidin–biotin assembly in a QDot-based assay was first documented in 2002 when Goldman et al. used avidin as a natural bridge between biotinylated antibody and QDots in a fluoroimmunoassay.[43] The avidin family proteins are capable of forming tight complexes with biotin, a small organic molecule, and includes streptavidin and similar, evolutionary unrelated, biotin-binding avidin

and neutravidin.[44] Streptavidin is a homotetrameric protein with a molecular mass of approximately 52.8 kDa; each streptavidin subunit is capable of binding one biotin molecule, resulting in one of the strongest and most stable noncovalent interactions in nature (dissociation constant, $K_d \sim 10^{-15}$ M; half-life, greater than several days).[45,46] Together with high binding specificity and minimal background, such properties have enabled the streptavidin–biotin assembly to become a tool of first choice for the QDot visualization of biological targets. In a typical experiment, live cells expressing the membrane protein of interest are first incubated with a biotinylated primary antibody specific for the extracellular epitope of that protein. Then, the unbound, biotinylated probe is washed away, and the cells are incubated with QDots coated with several covalently attached streptavidin molecules (Sav–QDots). After excess Sav–QDots are washed away, the cells can be immediately visualized (Figure 4(a)). Alternatively, one can use an 'antibody sandwich' method wherein a target-bound primary antibody is recognized by a biotinylated secondary antibody that is then captured by Sav–QDots. Also, one can simply use QDots covalently coupled to secondary antibodies via SPDP/SMCC methodology to recognize the target-bound primary antibody (Figure 4(b)). Since the use of several antibodies can add substantial bulk to the resulting QDot-antibody probe bound to the target protein, it is not uncommon for antibodies to be digested to yield smaller Fab fragments that retain the antigen-binding site; this becomes particularly important in size-sensitive experiments. Some experiments require that the biotinylated probe be conjugated to Sav–QDots and applied in a one-step labeling protocol; the formation of such a conjugate eliminates unwanted effects due to cellular stimulation with the biotinylated probe, or can allow the preparation of a monovalent QDot probe. Although antibody-based QDot labeling of neuronal receptors and transporters is popular in the field, Sav–QDots can also be used in conjunction with biotinylated peptides[47] and organic ligands.[20,48]

Each QDot conjugation approach described above is associated with distinct advantages and drawbacks; the suitability of each approach depends on the material/probe availability and experimental requirements. The direct conjugation approach usually results in smaller QDot conjugates and enables multicolor experiments wherein several targets may be monitored with several sizes of QDots preconjugated to distinct biospecific molecules. On the other hand, direct conjugation involves additional preparation, purification, and characterization steps; however, often such steps are easily accomplished through the use
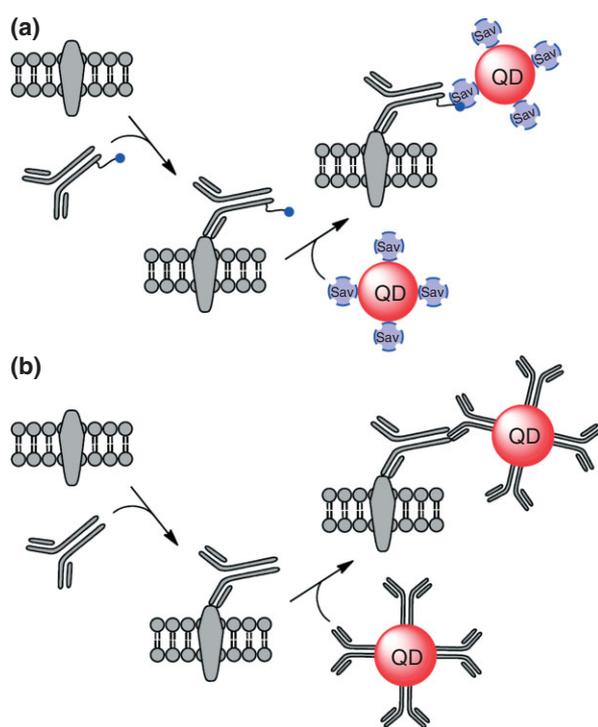
FIGURE 4 | Indirect Qdot labeling methodology. Indirect Qdot labeling typically involves either a streptavidin–biotin interaction between biotinylated probes and streptavidin-conjugated Qdots (a), or a noncovalent recognition of a target-bound primary antibody with secondary antibody-conjugated Qdots (b).

of commercially available QDot conjugation kits (Life Technologies). Also, direct conjugation usually results in the presence of several copies of probe at the QDot surface, with the conjugation stoichiometry being problematic to control. Depending on the application, such multivalent probe presentation at the QDot surface may lead to either cooperative binding and increased avidity or unwanted protein cross-linking due to multivalent binding.[49] In the indirect conjugation approach, the use of streptavidin significantly increases the final size of the QDot conjugates, an undesirable effect when it comes to size-sensitive QDot applications, such as fluorescence resonance energy transfer or *in vivo* imaging studies. Another issue with the use of the streptavidin–biotin assembly is the possibility of multivalent binding; for example, a commercially available Sav–QDot with a 655-nm emission maximum (Life Technologies) has approximately 5–10 streptavidin molecules at its surface. Each streptavidin has four biotin-binding sites, which results in a total of 20–40 biotin-binding sites per QDot. To address this issue, Howarth et al. genetically engineered a monovalent streptavidin protein with a single femtomolar affinity binding site.[50] Subsequently, a single copy of the monovalent streptavidin

was conjugated to the QDot surface to yield monovalent QDots capable of binding only a single copy of the biotinylated probe.[51] The advantages of the streptavidin–biotin approach include highly specific recognition of the biotinylated probe, ultralow nonspecific binding, and avoidance of time-consuming preparation steps, as Sav–QDots and biotinylated probes (antibodies) are readily available.[25]

QDots FOR BIOLOGICAL IMAGING OF NEURONAL RECEPTORS AND TRANSPORTERS

In the previous section, the selection of bioprobes and conjugation methods for the preparation of QDot nanoconjugates was discussed. In this section, specific examples that demonstrate how QDot nanoconjugates have been employed in recent molecular neuroscience studies are presented. A comprehensive list of the instances where QDots were used to target neuronal receptors and transporters has been compiled, and examples are classified based on the targeting probe and QDot conjugation strategy used (Table 2).

QDot Labeling of Neuronal Receptors and Transporters Using the Direct Conjugation Approach

As mentioned above QDots are typically synthesized in organic solvents and passivated with hydrophobic surfactants such as TOPO and TOP. Thus, one of the first methods used to generate ligand functionalized QDots for biological labeling was naturally the exchange of QDot hydrophobic surfactants with thiol (–SH) functionalized hydrophilic ligands.[8] In 2002, our group first employed the ligand exchange method in generating organic ligand functionalized QDots for neuronal transporter labeling[42] (Figure 5). In this study, a PEGylated, thiol-terminated serotonin ligand (5HT-PEG-SH) was exchanged with surface TOPO to yield water-soluble QDot-PEG-5HT conjugates, which were then used to image membrane serotonin transporters (SERTs) in living cells. Furthermore, these same QDot conjugates were employed in EC_{50} measurements and electrophysiological studies, demonstrating the potential utility of ligand-conjugated QDots in pharmacological and physiological applications.

Recently, owing to the popularity of high-quality, commercially available QDots, more scientists have begun to use these commercially available carboxyl- or amine-functionalized QDots to generate nanoconjugates for biological labeling. Although direct conjugation of biological probes to the these

TABLE 2 | Selected Examples of Quantum Dot Applications in the Studies of Neuronal Receptors and Transporters

Protein of Interest	Targeting Probe	QDot Conjugation Strategy	Cellular Expression Model	References
Serotonin transporter	Organic ligand	Ligand exchange	Transfected HEK293 cells	42
Serotonin transporter	Organic ligand	Biotin–Sav binding	mRNA-microinjected *Xenopus*	48
Serotonin transporter	Organic ligand	Biotin–Sav binding	Serotonergic RN46A cells	52
Dopamine transporter	Organic ligand	Biotin–Sav binding	Transfected flp-In 293 cells	20,53
GABAc receptor	Organic ligand	EDC coupling	mRNA-microinjected *Xenopus* oocytes	54
GABA$_A$ receptor (with GFP tag)	Anti-GFP antibody	Second antibody coupled to QDot	Hippocampal neurons	55
Glycine receptor	Antibody	Biotin–Sav binding	Primary rat spinal cord neurons	56,57
Glycine receptor	Antibody	SPDP/SMCC coupling	Transfected HeLa cells, primary neurons	58
Glial fibrillary acidic protein	Antibody	Biotin–Sav binding	Primary neurons and glia	59
TrkA and and P75 NGF receptors	βNGF peptide	Biotin–Sav binding	PC12 cells	60,61
5-HT$_{1A}$ receptor (with HA tag)	Anti-HA antibody	Biotin–Sav binding	N2a cells	62
AMPA receptor	Antibody	Second antibody coupled to QDot	Primary rat cortical neurons	63,64
AMPA receptor	Peptide	Second antibody coupled to QDot	Primary rat hippocampal neurons	65
Cannabinoid type 1 receptor	Antibody	Second antibody coupled to QDot	Primary rat hippocampal neurons	66
NMDA receptor	Antibody	Second antibody coupled to QDot	Primary rat hippocampal neurons	67
Metabotropic glutamate receptors (mGluR5)	Antibody	Biotin–Sav binding	Primary rat hippocampal neurons	68
Nicotinic acetylcholine receptor (nAChR)	Protein/toxin	Biotin–Sav binding	Neuromuscular synapses in the mouse diaphragm	47
Nicotinic acetylcholine receptor (nAChR)	Antibody; Protein/toxin	Biotin–Sav binding	Chick CG neurons	69
Angiotensin II receptor type 1	Peptide	EDC coupling	Transfected CHO cells	70
Presynaptic L-type calcium channel	Antibody	Biotin–Sav binding	Synapses of the tiger salamander retina	71
P2 purinergic receptors	Organic ligand	EDC coupling	PC12 cells	72
D2 dopamine receptor	Organic ligand	EDC coupling	Transfected A9 cells	73

QDots involves additional 'wet' chemistry techniques (see previous section), this preparation provides an additional degree of freedom in controlling how nanoconjugates interact with biological targets (e.g., adding a PEG chain to further decrease the cytotoxicity and non-specific binding[74]; or manipulating the probe multivalency[75]). In particular, PEGylated muscimol, a GABA$_C$ receptor agonist, was covalently conjugated to the QDot surface via EDC/NHS coupling.[54] In the follow-up experiments, it was demonstrated that ligand valency on GABA$_C$ affects its binding avidity and affinity.[75] Clarke et al. used a similar conjugation approach to design a redox-sensitive probe.[73] On the basis of the electron transfer mechanisms, dopamine-conjugated QDots were used to sense an intracellular oxidative state. Under reducing conditions, these QDot probes were only visible in the cell

periphery and lysosomes, whereas in mild oxidizing conditions, QDot fluorescence appeared in the perinuclear region. Under strongly oxidizing conditions, the QDot probe-associated fluorescence was visible throughout the cell. This experiment demonstrated the potential utility of QDot probes for sensing intracellular environments.

QDot Labeling of Neuronal Receptors and Transporters Using the Indirect Conjugation Approach

Shortly after Goldman et al. recognized the potential of the avidin family of proteins to facilitate the preparation of stable QDot–antibody conjugates,[43] Dahan et al. used the streptavidin–biotin assembly to detect single endogenous glycine receptor (GlyR)

(a)

(b)

FIGURE 5 | Ligand-conjugated QDots for SERT labeling in living cells. (a) Fluorescence images of SERT expressing HEK cells labeled with serotonin ligand functionalized QDots. Fluorescence labeling of SERT in the membrane is clearly visible (left) while cells preincubated with high-affinity SERT inhibitor paroxetine prior to the labeling shows no sign of fluorescence (right), indicating the QDot labeling specificity. (b) Electrophysiological currents elicited by serotonin or serotonin ligand functionalized QDots. The current induced by serotonin is typical. In contrast, the current induced by serotonin ligand functionalized QDots is characteristic of currents induced by SERT antagonists. (Reprinted with permission from Ref 42. Copyright 2002 American Chemical Society)

molecules at the surface of cultured rat spinal cord neurons.[56] The specific detection of GlyR α1 subunits was achieved through the use of a primary monoclonal antibody, a biotinylated Fab fragment of the secondary antibody, and Sav–QDots. Then QDots were used to visualize the lateral diffusion of single GlyR molecules. Importantly, this elegant study revealed that diffusion dynamics of GlyRs varies in synaptic, perisynaptic, and extrasynaptic domains of spinal neurons.

In 2008, Lévi et al. established that Ca^{2+}-driven excitatory synaptic transmission significantly restricted GlyR lateral diffusion and led to an increased subsequent clustering of GlyRs within the synaptic domain.[57] Furthermore, Charrier et al. demonstrated in 2010 that such a regulation of GlyR lateral diffusion at the excitatory synapses is mediated by β1 and β3 integrins, cell adhesion molecules, and signaling receptors that interact via calcium/calmodulin-dependent protein kinase II.[63] This progression is an impressive example of the evolution of QDots as fluorescent probes aimed at unraveling the molecular aspects of neuronal receptor regulation.

A similar 'sandwich' approach was also employed by Triller and colleagues to label γ-aminobutyric acid A receptors (GABA$_A$R). The γ2 subunit of the GABA$_A$R was sequentially labeled with an anti-γ2 antibody, a biotinylated secondary antibody, and then Sav–QDots. This approach was used by Bouzigues et al. to label GABA$_A$Rs in the growth cones (GCs) of rat spinal cord neurons.[76] In the presence of an extracellular GABA gradient, the authors showed that single QDot-labeled GABA$_A$ receptors redistribute asymmetrically across the GC, located at the axon tip, toward the gradient source

in a microtubule- and calcium-dependent manner. In 2009, Bannai et al. relied on a modified approach for GABA$_A$R labeling, in which a biotinylated secondary Fab fragment was used in conjunction with primary antibody and Sav–QDots. In this study, the authors demonstrated that GABA$_A$R diffusion coefficient and confinement domain size increase in response to enhanced excitatory synaptic activity.[77]

A distinct labeling approach based on streptavidin–biotin assembly was developed in the Ting Lab, where the authors adapted an enzymatic reaction to specifically biotinylate their proteins of interest.[78] In their design, a 15 amino acid acceptor peptide sequence (AP) was genetically fused to a C- or N-terminus of the protein, and a bacterial enzyme, biotin ligase (BirA), was used to biotinylate a lysine side chain within the AP sequence. Howarth et al. applied this approach to label AP-fused α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors in hippocampal neurons and then studied AMPA receptor synaptic localization.[65]

Cui et al. also used the biotin–streptavidin approach to prepare QDot nanoconjugates to label nerve growth factor (NGF) receptors in live PC12 and rat dorsal root ganglion (DRG) neurons (Figure 6).[79] In their study, biotinylated NGF peptides were preconjugated to Sav–QDots with a stoichiometric ratio of NGF to QDot of 1:1.2 to achieve a monovalent presentation of NGF dimer on the QDot surface. In a clever setup, NGF–QDot conjugates were first added to the microfluidic chamber containing distal axons of DRG neurons and allowed to bind and form complexes with NGF receptors and undergo subsequent internalization into early endosomes. Endosomes containing NGF–QDots were then demonstrated to exhibit 'stop-and-go' retrograde
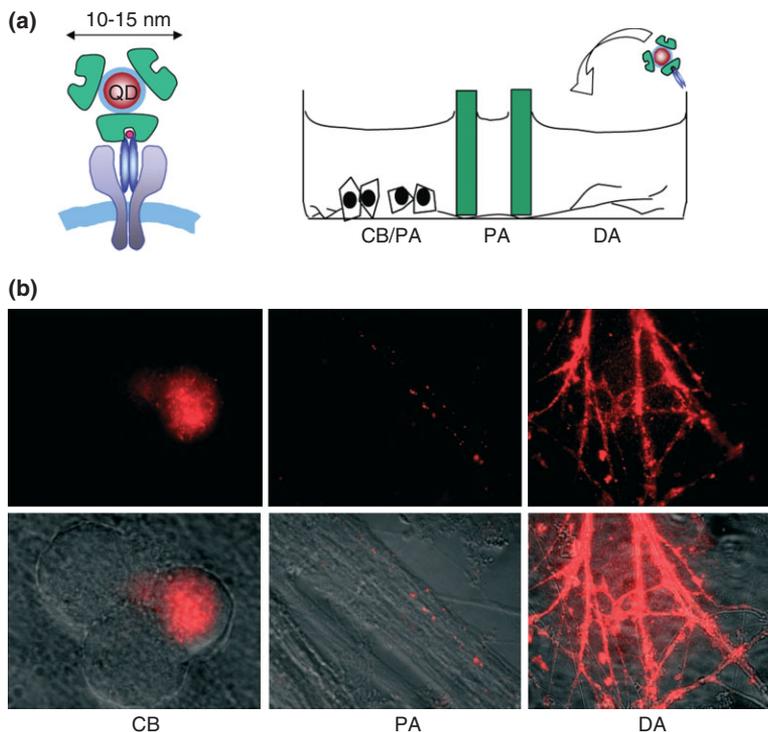
FIGURE 6 | QDot labeling of NGF receptors using streptavidin–biotin assembly. NGF-conjugated QDots were used to investigate retrograde, vesicular NGF transport in living DRG neurons. (a) Schematic drawing of a QDot–NGF bound to dimerized TrkA receptors (left) and addition of QDot–NGF to the DA compartment of the three-chamber DRG neuron culture (right). DA, distal axon; PA, proximal axon; CB, cell body. (b) Representative live fluorescence images of DRG neuron axons or cell bodies 2 h after the addition of 4 nM QDot–NGF to the DA chamber. QDot–NGF seems to bind all axons in distal axon chamber. However, only a small portion of the cell bodies and proximal axons are shown to have QDot fluorescence, reflecting the fact that not all cell bodies extend their axons into the distal axon compartment. (Reprinted with permission from Ref 79. Copyright 2007 National Academy of Sciences, USA)

transport toward the neuronal cell body with an average speed of $1.31 \pm 0.03\,\mu\mathrm{m}$ second^{-1}. Similar to Cui et al.,[79] Fichter et al. employed the preconjugation strategy to link a biotinylated anti-hemagglutinin (HA) antibody to Sav–QDot. The resulting QDot conjugates were used to label HA-fused serotonin receptor subtype 1A (5-HT$_{1A}$) and investigate the kinetics of receptor-mediated internalization of QDots.[62]

Our group has also previously utilized a biotin–streptavidin assembly strategy to label cell surface neuronal receptors and transporters with Sav–Q-Dots. In 2008, Orndorff et al. used a biotinylated α-bungarotoxin (α-BTX) and Sav–QDots in a two-step labeling protocol to visualize nicotinic acetylcholine receptors (nAChRs) on the surface of mouse diaphragm neuromuscular junction *ex vivo*.[47] The high affinity and specificity of α-BTX for nAChRs and ultralow nonspecific binding of Sav–QDots allowed direct assessment of the presence of endogenous nAChRs in native tissue. Such a labeling approach was later used by Fernandes et al. to investigate the lateral diffusion of endogenous nAChRs on the surface of chick ciliary ganglion neurons.[80] In a study using the presynaptic dopamine transporter (DAT) expressed in live HeLa and HEK-293 Flp-In cells, Kovtun et al. targeted DAT with a biotinylated ligand, 2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane (IDT444), that can be bound by Sav–QDots[20] (Figure 7). The lack of an efficient antibody against

the extracellular DAT epitope required that fairly sophisticated organic chemistry be employed to facilitate QDot labeling of DAT, whereby the cocaine analogue β-CFT was conjugated to a biotinylated, flexible linker arm to enable subsequent QDot recognition. QDot-IDT444 probes were demonstrated to possess high affinity for DAT, as evidenced by the IC$_{50}$ (half-maximal inhibitory concentration) value of roughly 50 nM. The specific interaction of these QDot probes with the DAT primary binding site was completely blocked by high-affinity DAT antagonist, GBR12909. Moreover, the binding interaction was shown to be virtually irreversible, as DAT-bound QDots were not readily displaced after prolonged exposure of QDot-labeled cell cultures to cocaine derivatives and GBR12909. In addition, QDot-labeled membrane DATs were demonstrated to undergo acute internalization in response to protein kinase C (PKC) activation with phorbol 12-myristate 13-acetate (PMA). PKC-mediated phosphorylation of QDot-labeled DATs led to endosomal accumulation of DAT–QDot complexes as evidenced by the appearance of punctate intracellular fluorescence after 30 min of PMA incubation (Figure 7(B1)). In contrast, the QDot-associated fluorescence remained predominantly on the cell surface in the control group exposed to dimethyl sulfoxide (DMSO/vehicle) only (Figure 7(A1)). In a follow-up set of experiments, Kovtun et al. took advantage of excellent specificity,

FIGURE 7 | Labeling of dopamine transporter (DAT) with ligand-conjugated QDots in live HeLa cells. (left) streptavidin-conjugated QDots were used to label DATs previously exposed to a biotinylated, PEGylated cocaine analog. (upright) chemical structure of the DAT ligand used in the study. (A1) QDot labeling of membrane DATs in a live HeLa cell. (B1) QDot-bound DATs underwent acute redistribution from the plasma membrane to intracellular compartments as a result of protein kinase C (PKC) activation. (Reprinted with permission from Ref 20. Copyright 2011 American Chemical Society)
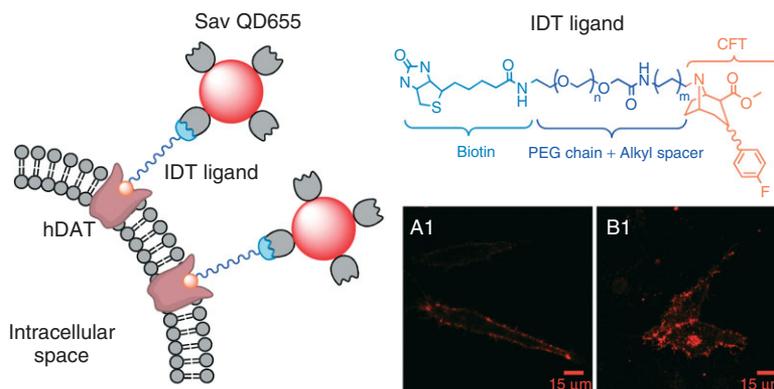
high-affinity, and temporally stable binding interaction of these antagonist-conjugated QDots to develop a QDot-based DAT binding assay.[53] The use of flow cytometry enabled rapid, multiwell analysis of large QDot-labeled cell populations at the single-cell level. As a proof-of-concept, the QDot-based DAT binding assay was used to accurately measure inhibitory action of known DAT modulators; dose–response curves of median QDot fluorescence intensity per cell were generated for titrations of DAT-expressing HEK293 cells with PMA and GBR12909. Kovtun et al. anticipated that the reported QDot-based binding assay would be of immediate value to the high-throughput screening (HTS) of novel small-molecule modulators of DAT function.

Recently, Chang et al. went a step further to adapt the QDot nanoconjugates for antidepressant drug screening[48] (Figure 8). Our approach targeted human SERT (hSERT), the primary target for drugs used to treat major depression. A custom-synthesized, QDot-tagged indoleamine derivative that is structurally similar to 5-HT and that can access the primary neurotransmitter binding site of hSERT was employed to demonstrate that interactions between QDot-tagged ligand and hSERT could be displaced by other antidepressants, providing an opportunity for a screen for allosteric, hSERT-targeted small molecules. Another area of ongoing investigation surrounding SERTs in our group concerns the membrane dynamics of single SERTs in response to their natural signaling stimuli. In a study using serotonergic RN46A cells, Chang et al. implement the ligand-conjugated QDot labeling approach to monitor single SERT proteins.[52] Two modes of SERT membrane dynamics were identified in the study, in which the majority of QDot-labeled single SERTs were constrained in cholesterol-rich membrane microdomains (often referred to as lipid rafts), and a small fraction showed relatively free diffusion in the plasma membrane. The diffusion dynamics of single SERTs and the
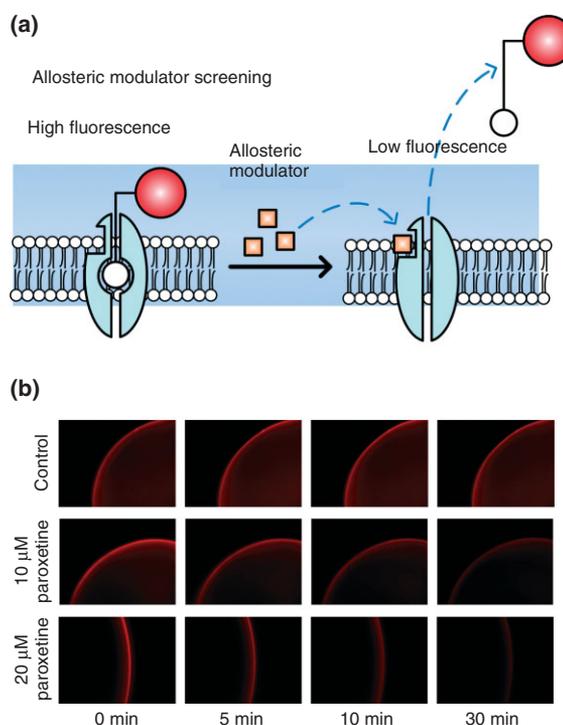

FIGURE 8 | Fluorescence displacement assay based on ligand-conjugated QDots for the drug discovery of allosteric antidepressants. (a) Targeted hSERTs bind to the QDot-tagged ligands, forming complexes that increase fluorescent signal along the membrane. When exposed to a potential drug that induces a conformational change in the binding site, the QDot-tagged ligands are displaced resulting in a decrease in fluorescence intensity. (b) Representative time-lapse fluorescent images show the effect of paroxetine on ligand-hSERT displacement in the presence of PBS buffer (control), 10 μM, and 20 μM paroxetine. (Reprinted with permission from Ref 65. Copyright 2011 American Chemical Society)

relationship of these dynamics to membrane compartments and regulatory stimuli, including cGMP and IL-1β, were also investigated. Notably, single SERTs activated by 8-bromo-cGMP and IL-1β remain colocalized with membrane microdomains, leading

to the hypothesis that cholesterol-rich membrane microdomains may have essential roles in SERT regulation. This could be the first direct observation of single SERT membrane dynamics in real-time in a living cell.

CONCLUSIONS AND FUTURE PERSPECTIVES

In the past two decades, technological advances in QDot synthesis have advanced opportunities for fluorescence-based biological imaging.[14] Currently available QDot probes are characterized by minimal cytotoxicity, improved stability in biological environments, and ultralow nonspecific binding. Most importantly, advances in surface chemistry have allowed for the preservation of key QDot optical properties, including high quantum yield, large Stokes shift, and narrow fluorescence emission spectra. These advances have prompted a significant increase in the use of Qdots by molecular and cellular neuroscientists. The most important achievement of QDot technology in molecular neuroscience is likely to be the use of this approach for single-QDot tracking techniques to investigate membrane protein dynamics in neuronal cells at the single-molecule level.[81,82] As mentioned above, Dahan et al. were the first to utilize this tool to interrogate the diffusion dynamics of individual GlyRs.[56] Similar approaches were subsequently employed to investigate various neuronal signaling related targets including NGF,[79] glial fibrillary acidic protein,[59] and GABA$_A$R.[77] In addition, several solutions have been suggested to overcome the blinking shortcoming for Qdot tracking experiments, including single QDot blinking suppression,[29] and synthesis of nonblinking colloidal QDots.[31] However, rendering the newly generated QDots biocompatible and readily adaptable to neuroscience research is not a trivial matter, requiring an integration of multiple disciplines, including chemistry, material science, and neurobiology. With this in mind, we expect to witness increasing collaboration among various scientific disciplines toward the pursuit of better QDot reporters for the field of neuroscience.

Another promising application of QDot nanoconjugates in molecular neuroscience is exertion of specific physiological effects as a result of the interaction with protein targets and neuronal cells and subsequent uncovering of the underlying neuronal signaling mechanisms. However, current QDot applications in neuroscience are mostly limited to revealing the position of the protein target without further extending into the use of QDot nanoconjugates for stimulation of cellular responses/processes. The potential of this approach has recently been demonstrated through the use of QDot nanoconjugates functionalized with biologically active organic ligands and small peptides. Specifically, our serotonin-conjugated QDots displayed target-specific SERT labeling and, importantly, effectively retained pharmacological and physiological properties of native serotonin.[42] Another fine example was demonstrated by Vu et al. wherein NGF-functionalized QDots were employed to stimulate the neuronal differentiation in PC12 cells.[60]

In conclusion, as with any new technology, there is always a period of time before the technology matures and begins to bear fruit. To fully explore the tremendous potential that QDots offer to the field of neuroscience, collaborations of researchers representing a diverse range of disciplines, including chemistry, material science, neuroscience, pharmacology, and medicine, are required. With the continuous advances in QDot synthesis in parallel with the improvements in QDot bioconjugation protocols, we envision it will not be long before the QDot-based biological labeling techniques are considered routine methods in a standard neuroscience laboratory.

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