Published in final edited form as:

J Am Chem Soc. 2006 December 13; 128(49): 15701–15713. doi:10.1021/ja064324k.

BINDING OF MUSCIMOL-CONJUGATED QUANTUM DOTS TO GABA_C RECEPTORS

Hélène A. Gussin[†], Ian D. Tomlinson[‡], Deborah M. Little[#], Michael R. Warnement[‡], Haohua Qian[†], Sandra J. Rosenthal[‡], and David R. Pepperberg^{†,*}

[†] *Lions of Illinois Eye Research Institute, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, IL 60612*

[#] *Department of Neurology and Rehabilitation, and Center for Cognitive Medicine, University of Illinois at Chicago, Chicago, IL 60612*

[‡] *Department of Chemistry, Vanderbilt University, Nashville, TN 37232*

Abstract

Functionalization of highly fluorescent CdSe/ZnS core-shell nanocrystals (quantum dots, qdots) is an emerging technology for labeling cell surface proteins. We have synthesized a conjugate consisting of ~150–200 muscimols (a GABA receptor agonist) covalently joined to the qdot via a poly(ethylene glycol) (PEG) linker (~78 ethylene glycol units), and investigated the binding of this muscimol-PEG-qdot conjugate to homomeric $\rho 1$ GABA_C receptors expressed in *Xenopus* oocytes. GABA_C receptors mediate inhibitory synaptic signaling at multiple locations in the CNS. Binding of the conjugate was analyzed quantitatively by determining the fluorescence intensity of the oocyte surface membrane in relation to that of the surrounding incubation medium. Upon 5- to 10-min incubation with muscimol-PEG-qdots (34 nM in qdot concentration), GABA_C-expressing oocytes exhibited a fluorescent halo at the surface membrane that significantly exceeded the fluorescence of the incubation medium. This halo was absent following muscimol-PEG-qdot treatment of oocytes lacking GABA_C receptors. Incubation of the oocyte with free muscimol (100 μ M – 5 mM), PEG-muscimol (500 μ M) or GABA (100 μ M – 5 mM) substantially reduced or eliminated the fluorescence halo produced by muscimol-PEG-qdots, and the removal of GABA or free muscimol led to a recovery of muscimol-PEG-qdot binding. Unconjugated qdots and PEG-qdots that lacked conjugated muscimol neither exhibited significant binding activity nor diminished the subsequent binding of muscimol-PEG-qdots. The results indicate that muscimol joined to qdots via a long-chain PEG linker exhibits specific binding activity at the ligand-binding pocket of expressed GABA_C receptors, despite the presence of both the long PEG linker and the sterically bulky qdot.

Keywords

quantum dot; nanocrystal; GABA_C receptor; ligand-gated ion channel; muscimol; GABA

INTRODUCTION

Fluorescent nanocrystals, or quantum dots (qdots), have shown great promise as biological imaging agents since their introduction in 1998.^{1,2} They have many inherent advantages over conventional fluorophores.^{2–5} Specifically, their higher quantum yields, photo stability, size-

*Corresponding author: Dr. David R. Pepperberg, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W. Taylor Street, Chicago, IL 60612, Phone: 312-996-4262; Fax: 312-996-7773, Corresponding author's email address: davipepp@uic.edu.

tunable, narrow emission spectra and continuous absorption spectrum make them ideally suited for highly sensitive, multiplexed, dynamic imaging. Various examples of biological imaging with qdots have been reported,⁶ including whole cell assays² and imaging applications *in vivo*.⁷

For efficient biolabeling by a qdot-containing preparation, the qdots must maintain three properties under aqueous conditions: efficient fluorescence, colloidal stability and low nonspecific adsorption. A variety of techniques have been used to achieve these goals, including encapsulation in micelles,⁸ silanization,⁹ encapsulation in amphiphilic polymers^{10,11} and encapsulation in proteins such as avidin and streptavidin.¹² Biological activity is introduced by conjugating ligands that have the desired biological effect. A variety of biologically active ligands have been linked to qdots, such as proteins,^{13–18} peptides^{19,20} and antibodies.^{11,12,21–23} The most common method used for this conjugation is the attachment of biotin to the biologically active molecule, followed by attachment of the biotinylated ligand to streptavidin-coated qdots. For example, Dahan *et al.*²³ have reported the use of biotinylated antibodies and streptavidin-qdots, to target glycine receptors and achieve dynamic imaging of live cells.

A distinct approach to achieving bioactivity of a qdot-containing structure is to synthesize derivatives of biologically active small molecules^{24–27} and attach them to the qdot. Neurotransmitters and other small molecules that interact with cells of the central nervous system (CNS) are of particular interest in this regard. In an earlier study, specific labeling of the serotonin transporter protein using serotonin-conjugated qdots was demonstrated.²⁸ The present study further develops the use of small molecule-conjugated qdots as probes for postsynaptic membrane receptors. Specifically, we have employed quantitative fluorescence imaging to examine the binding, to neurotransmitter receptors expressed in a model cell system, of a novel qdot conjugate containing an agonist for the receptor. In the investigated qdot conjugate, the receptor agonist is joined, via a poly(ethylene glycol) (PEG) chain and an amide linkage, to CdSe/ZnS core-shell qdots coated with a modified polyacrylamide (AMP[®]) polymer. Use of the PEG linker was motivated by earlier findings²⁹ indicating that PEGylation of the qdot reduces nonspecific binding of the resulting conjugate to cell surfaces.

The neurotransmitter receptor selected for the present investigation is the GABA_C receptor, a ligand-gated ion channel that *in vivo* is activated by the neurotransmitter γ -aminobutyric acid (GABA). GABA_C receptors are expressed in the retina and in many other regions of the CNS.^{30–36} Native GABA_C receptors are pentameric and consist of a heteromeric combination of GABA ρ subunits.^{37–39} However, GABA_C subunits are capable of forming fully functional homopentamers when expressed in model cell systems such as *Xenopus* oocytes;^{40–42} the homopentamer structure is of advantage in that it simplifies both receptor expression and the analysis of binding. Muscimol (5-aminomethyl-3-hydroxyisoxazole) is a well known agonist of GABA_C receptors (as well as of GABA_A receptors, another GABA-responsive postsynaptic receptor widely distributed in the CNS). The present experiments show that a conjugate consisting of muscimol joined to AMP[®] qdots through an aminohexanoyl linker and PEG 3400 chain exhibits robust and specific binding to GABA_C receptors expressed in *Xenopus* oocytes.

EXPERIMENTAL SECTION

Chemical synthesis and characterization

Materials—AMP[®]-coated CdSe/ZnS core-shell qdots with maximum fluorescence emission at 605 nm were a gift from Quantum Dot Corporation (Haywood, CA) and were supplied as an 8.5 μ M solution in borate buffer (pH 8.5). The same AMP-coated qdots are now available from Invitrogen (Invitrogen Corporation, Carlsbad, CA) and referred to as the Qdot Innovator's Tool Kit ITK nanocrystals. *t*-Butyloxycarbonyl (BOC) amine-poly(ethylene glycol)-*N*-

hydroxysuccinimide ester (BOC-NH-PEG-NHS; purity >85%) was obtained from Nektar Therapeutics (Huntsville, AL). The poly(ethylene glycol) (PEG) chains of this product had a molecular weight (determined by MALDI) of 3446 Da (approximately 78 ethylene glycol units) and a polydispersity of 1.00. Unless otherwise indicated, the term “PEG” used throughout the present text denotes a PEG chain consisting of about 78 ethylene glycol units. Trifluoroacetic acid (TFA), *N*-hydroxy succinimide (NHS), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), *p*-toluene sulfonyl chloride, hydrazine hydrate and pyridine were obtained from Aldrich Chemical Corporation (Milwaukee, WI). Methylene chloride, acetonitrile, ethanol and methanol were obtained from Fischer Scientific (Fair Lawn, NJ) and used without purification. Potassium phthalimide was obtained from Lancaster Synthesis (Windham, NH). Sodium hydroxide and magnesium sulfate were obtained from VWR International (West Chester, PA). PEG 2000 monomethyl ether [poly(ethylene glycol) chains of molecular weight ~2000 Da on average (polydispersity of 1.07; molecular weight range of 1895 – 2167 Da)] was obtained from Fluka/Sigma-Aldrich, St. Louis, MO. Borate buffer was obtained from Poly Sciences Inc. (Warrington, PA) as a 5X concentrate and diluted to 1X before use. Sephadex G-50 was obtained from Amersham Biosciences (Uppsala, Sweden). All other reagents were used as supplied unless otherwise stated.

Overview of preparation of qdot-conjugates—The primary structure synthesized for investigation was a CdSe/ZnS qdot preparation functionalized to contain multiple copies of the GABA_C agonist muscimol. Each muscimol group was joined to the qdot through a short (6-aminohexanoyl; AH) spacer and a PEG 3400 linker (Fig. 1). Muscimol, synthesized as described by Frey and Jäger,⁴³ was attached to the AH spacer by reaction with 6-*tert*-butoxycarbonyl-amino-hexanoic acid *N*-hydroxysuccinimide ester (BOC-AH-NHS, synthesized as described by Doughty *et al.*⁴⁴), yielding **compound 1** in Fig. 2. After removal of the BOC protecting group with TFA, the resulting AH-muscimol (**2**) was coupled to BOC-NH-PEG-CONHS. Removal of the BOC group of the product (**3**) with TFA provided NH₂-PEG-AH-muscimol (**4**), henceforth termed “PEG-muscimol”. The PEG-muscimol was conjugated to terminating carboxyl groups on the AMP-coated qdots using 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), after which the conjugate, henceforth referred to as M-PEG-qdot (muscimol-PEG-qdot), was purified by size exclusion chromatography (Sephadex G-50) and analyzed for concentration by absorbance spectrophotometry. We also prepared a qdot conjugate consisting of methyl-terminated PEG 2000 conjugated to qdots, i.e., a conjugate that lacked the terminating amino-hexanoyl muscimol of the primary structure described in Fig. 1. This preparation, henceforth referred to as PEG-qdots, was prepared using a Gabriel synthesis, as follows. Commercially obtained monomethoxy terminated PEG 2000 was converted to monoaminomethoxy terminated PEG 2000 by converting the terminal hydroxyl functionality to a tosylate (**5**). The tosylate was then displaced by refluxing it in acetonitrile with potassium phthalimide, yielding (**6**). Finally, the phthalimide was converted to the amine (**7**) using hydrazine monohydrate. The following paragraphs describe details for of the synthesis and analysis of structures **1–7** noted above.

{5-[(3-Hydroxy-isoxazol-5-ylmethyl)-carbamoyl]-pentyl}-carbamic acid tertbutyl ester (1**)**—Muscimol (0.1 g, 0.88 mmol) and 6-*tert*-butoxycarbonylamino-hexanoic acid 2,5-dioxo-pyrrolidin-1-yl ester (0.29 g, 0.88 mmol) were dissolved in pyridine (5 ml), and the mixture was stirred at room temperature for 18 hr. After evaporation, the product was purified via column chromatography on silica gel and eluted with methylene chloride (92%): methanol (8%). The partially purified compound was further purified by washing with diethyl ether (10 × 10 ml). This yielded 0.15 g (54%) of the desired product as a colorless solid (melting point: = 135–136 °C). ¹H NMR (Acetone-d₆) 1.34–1.49 (m, 13H), 1.58–1.67 (m, 2H), 2.53 (t, 2H), 3.06 (q, 2H), 4.38 (d, 2H), 5.86 (s, 1H), 5.97 (s, 1H), 7.68 (s, 1H), 9.90 (brs, 1H).

6-amino-hexanoic acid [3-hydroxy-isoxazol-5-ylmethyl]-amide (2)—{5-[(3-Hydroxy-isoxazol-5-ylmethyl)-carbamoyl]-pentyl}-carbamic acid *tert*butyl ester (0.2 g, 0.64 mmol) was dissolved in 95% TFA (2 ml) and stirred at room temperature for 1 hr. The solution was evaporated and dried under reduced pressure for 4 days. The crude product was used without further purification. ¹H NMR (DMSO-*d*₆) 1.14–1.15 (m, 2H), 1.68–1.69 (m, 2H), 1.83–1.84 (m, 2H), 2.30–2.33 (m, 2H), 2.59 (s, 2H), 3.85 (s, 2H), 4.40 (d, 2H), 5.90 (s, 1H), 8.00 (s, 1H), 9.95 (s, 1H).

BOC- protected muscimol derivative (3)—0.2 g of *t*-butyloxycarbonyl (BOC) amine-PEG-activated acid (BOC-NH-PEG-NHS) was weighed out in a round-bottomed flask and pyridine (2 ml) was added. 6-Amino-hexanoic acid [3-hydroxy-isoxazol-5-ylmethyl]-amide (0.04 g, 0.01 mmol) was dissolved in pyridine (1 ml) and added. The mixture was stirred at room temperature for 18 hr, then evaporated under reduced pressure. The crude product was washed with diethyl ether (5 × 20 ml) and dried under reduced pressure. The resulting product was obtained as a brown tar. MALDI mass spectroscopy confirmed that the PEG had reacted with the 6-amino-hexanoic acid [3-hydroxy-isoxazol-5-ylmethyl]-amide, and this product was used without further purification. ¹H NMR (Acetone-*d*₆) 1.41–1.48 (m, 11H), 1.60–1.68 (m, 2H), 2.20–2.25 (m, 2H), 2.49 (t, 2H), 3.20–3.21 (m, 2H), 3.60 (nH), 4.40 (d, 2H), 5.91 (s, 1H), 5.95 (s, 1H), 8.01 (s, 1H).

Deprotected muscimol (4)—The BOC-protected muscimol compound was stirred in a mixture of 95% TFA (5 ml) and methylene chloride (5 ml) for 1 hr, and the solvent was removed by evaporation under reduced pressure. The product was washed with diethyl ether (5 × 20 ml) then dried under reduced pressure for 1 week. MALDI mass spectroscopy confirmed that the BOC group had been removed. After drying for 1 week, the product was obtained as a tar that was used without further purification.

Monomethoxy tosyl PEG 2000 (5)—PEG 2000 monomethyl ether (20 g) was dissolved in methylene chloride (200 ml) and cooled to 0 °C in an ice/acetone bath. Para toluene sulfonylchloride (2 g, 0.01 mol) was added and the mixture was stirred at 0 °C for 30 min. Freshly powdered potassium hydroxide (4.5 g, 0.08 mol) was added, and the mixture was stirred at room temperature for 6 hr. After warming to room temperature, the solution was washed with water (2 × 20 ml) and dried over magnesium sulfate. The methylene chloride was removed under reduced pressure, and the product purified by washing with hexanes (5 × 200 ml). This yielded 18 g of the product as a waxy solid ¹H NMR (CDCl₃) δ 2.42 (s, 3H), 3.39 (s, 3H), 3.60 (s, nH), 3.82–3.86(m, 2H), 4.10–4.14 (m, 2H), 7.31 (d, 2ArH), 7.75 (d, 2 ArH).

Monophthalimido PEG 2000 monomethyl ether (6)—Monotosyl PEG 2000 monomethyl ether (18 g) and potassium phthalimide (1.31 g) were dissolved in acetonitrile (200 ml) and heated at reflux with stirring for 18 hr. The mixture was cooled and evaporated, and dissolved in methylene chloride (200 ml). The organic solution was washed with water (2 × 50 ml) and dried over magnesium sulfate. After filtering and evaporating under reduced pressure, the crude product was purified by washing with hexanes (5 × 200 ml). This yielded 7.7 g of the product (**6**) as a colorless wax. ¹H NMR (CDCl₃) δ 3.36 (s, 3H), 3.52 (s, nH), 3.68 (m, 2H), 3.82 (m, 2H), 7.69 (m, 2ArH), 7.79 (m, 2ArH).

Monoamino PEG 2000 monomethyl ether (7)—Monophthalimido PEG 2000 monomethyl ether (7.7 g) was dissolved in ethanol (200 ml) and hydrazine monohydrate (10ml) was added. The mixture was stirred at room temperature for 18 hr and then evaporated under reduced pressure. The crude product was dissolved in methylene chloride and stirred for 18 hr at room temperature. The solution was filtered and washed with water (2 × 50 ml) and dried over magnesium sulfate. After filtration and evaporation, the product was purified by washing

with hexanes (5×200 ml). This yielded the product (**7**) as a colorless wax (5.9g). ^1H NMR (CDCl_3) δ 3.32 (s, 3H), 3.56 (s, nH), 3.69 (m, 2H).

Derivatization of qdots with structures (**4**) and (**7**)

Muscimol-containing structure (4): 0.1 ml of an $8.4 \mu\text{M}$ solution of qdots was placed in a vial equipped with a stir bar. To this was added 0.3 ml of borate buffer (pH 8.5) that contained 3 mg (1000 equivalents) of the muscimol-containing compound (**4**). Then *N*-hydroxy succinimide (72 μg , 0.63 μmol) dissolved in borate buffer (0.1 ml) was added. This was followed by the addition of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (0.12 mg, 0.63 μmol) dissolved in borate buffer (0.1 ml). The mixture was stirred at room temperature for 2 hr, then purified by passage through a Sephadex G-50 column and eluted with borate buffer (pH 8.5). The fluorescent fractions were combined to yield the purified muscimol-functionalized qdots (M-PEG-qdots). Fig. 3A shows a UV-visible spectrum of the M-PEG-qdots. The spectrum is dominated by the absorbance of the qdot itself, exhibiting a peak near 600 nm indicative of the first excited state. The absorbance continuously increases at shorter wavelengths due to excitation of higher lying electronic states. This spectral line shape is typical for quantum dots. The spectrum blue-shifts for smaller dots and red-shifts for larger dots owing to “particle-in-a-box” quantum mechanical properties.⁴⁵ The concentration of qdots in this preparation was determined based on a molar extinction coefficient of $6.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 600 nm (private communication, M. Bruchez). The derivatization of qdots with the muscimol- and PEG 3400-containing structure (**4**) and with the PEG 2000-containing structure (**7**) that lacked muscimol was evaluated by electrophoresis in 1% agarose gels using Tris-acetate-EDTA (TAB) buffer, pH 7.4 (Fig. 3B). Underivatized AMP-coated qdots were used as a control. The evident streaking of the M-PEG-qdot conjugate is attributed to variability in loading of the qdots with the muscimol-containing structure. Agarose gel electrophoresis of qdots derivatized with the PEG 2000-containing structure (**7**) also showed that these qdots were functionalized. The coupling efficiency for the conjugation of structure (**7**) to AMP qdots has previously been observed to be $\sim 20\%$.²⁹ As the present muscimol-containing M-PEG-qdot (Fig. 1) is a derivative of PEG 3400, a similar coupling efficiency is likely. Hence, each qdot of the M-PEG-qdot was presumed to be derivatized with approximately 150–200 muscimol groups on average. Conjugation was attempted using a much higher (2,000-fold) molar excess of the muscimol-containing structure, but this higher loading condition led to precipitation of the conjugates. Qdots conjugated with 1000 equivalents and 2000 equivalents of the muscimol ligand were imaged using an inverted microscope. These images clearly show that the qdots had formed a precipitate when 2000 equivalents were used. However, when 1000 equivalents were used, no precipitation was observed (see Supporting Information). We attribute this precipitation observed with high loading as due either to increased lipophilicity of the resulting conjugate or to the formation of multiple hydrogen bonds between muscimol groups on different qdots. In addition to precipitation, higher loading with the muscimol-containing structure led to a significant quenching of qdot fluorescence. Such a quenching phenomenon has previously been observed²⁹ and attributed to the ratios of EDC and NHS used for preparation of the conjugate. Quantum yields were measured for the unconjugated AMP-coated qdots and qdots conjugated with 1000 equivalents of muscimol ligand and found to be 0.297 and 0.137, respectively, for these materials (see Supporting Information). Both the AMP-coated qdots and the muscimol conjugates showed robust fluorescence, and exhibited little photobleaching over a time course of 1 hr (Supporting Information).

Monoamino monomethyl ether (7): 0.1 ml of a solution containing 2000 equivalents of (**7**) in borate buffer at pH 8.5 was placed in a reaction vial equipped with a magnetic stirrer. To this was added 0.1 ml of a borate solution containing 1500 equivalents of NHS, 0.1 ml of an $8.5 \mu\text{M}$ solution of qdots in borate buffer, and 0.1 ml of a solution containing 1500 equivalents of EDC in borate buffer. This mixture was stirred for 2 hr at room temperature, and the

PEGylated qdots were purified by size exclusion chromatography on Sephadex G-50, yielding the conjugate PEG 2000 with the qdots (PEG-qdots). The concentration of the qdot preparation was determined by UV-visible spectroscopy, using an extinction coefficient of $6.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. By analogy with the considerations noted above for the M-PEG-qdot preparation, we estimate that average loading in the PEG-qdot preparation was ~300–400 PEG 2000s per qdot.

MALDI TOF mass spectroscopy—Compounds (3) and (4) were characterized using MALDI TOF mass spectroscopy. MALDI-TOF mass spectra were recorded on an Applied Biosystems Voyager mass spectrometer equipped with a 337 nm nitrogen laser. The acceleration voltage was 25 kV, and 30 to 64 scans were averaged for each spectrum. For sample preparation, a saturated matrix stock solution of 2,5-dihydroxybenzoic acid and a 0.01 M sodium iodide solution were prepared in methanol. Stock solutions of polyethylene glycol amine (5 mM) in methanol and the polyethylene glycol conjugates in water were prepared. The stock solutions were mixed in a 2:5:2 ratio of sample to matrix to salt by volume. A 1 μl aliquot of each sample solution was placed on the sample plate. A PEG standard prepared in the same manner as the other samples was used for mass calibration of the instrument. The resulting spectra indicated that compounds (3) and (4) were polydisperse. Compound (3) exhibited masses ranging from 3241 Da to 4188 Da, indicating the conjugation of muscimol to PEGs of different lengths. The peak of greatest intensity had a mass of 3726 Da. When compound (3) was treated with TFA to yield compound (4), the observed masses in the MALDI TOF spectrum shifted by 100 Da, corresponding to loss of the BOC protecting group, and the most intense peak was observed to have a mass of 2626 Da. In addition, a MALDI TOF mass spectrum obtained for compound (7) was found to be consistent with the desired structure.

Oocyte preparation and receptor expression

All animal procedures adhered to institutional policies and to the Statement for the Use of Animals in Ophthalmic and Vision Research adopted by the Association for Research in Vision and Ophthalmology (ARVO). Ovarian lobes of gravid adult female *Xenopus laevis* toads (Xenopus One, Ann Arbor, MI), anaesthetized with MS-222 (1 g/l), were excised and the oocytes were removed. Stages V–VI oocytes were selected and stored in frog Ringer solution (100mM NaCl, 2mM KCl, 2mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose and 5 mM HEPES, pH 7.4). The follicular layer was removed by 30 min-immersion of the oocytes in Ca^{2+} -free Ringer solution containing 2 mg/ml collagenase, at room temperature. The expression of GABA_C receptors (human $\rho 1$ and perch $\rho 1B$) in *Xenopus laevis* oocytes was achieved using previously described procedures.^{40,46} Fifty nl of cRNA, obtained for each GABA_C receptor subunit from *in vitro* transcription (mMessage mMachine Ambion Inc., Austin, TX) from linearized cDNAs, was injected into the oocytes (Drummond Nanoject II; Drummond Scientific Co., Broomall, PA). Oocytes were assayed after 18–72 hr storage in Ringer solution containing 0.1 mg/ml gentamycin at 16–19 °C, to allow for expression of the GABA_C receptors. Control oocytes were obtained by either leaving the oocytes uninjected, or by injecting water only rather than cRNA solution.

Confocal microscopy

Images were obtained from oocytes positioned in a glass-bottom dish and incubated for defined periods in Ringer solution supplemented with one or more test components. Experiments were conducted at ambient temperature (range: 16–20 °C). To prevent degradation of the oocyte, the maximum duration of an experiment was 60 min. After each incubation or washing step, the oocytes were visually inspected to detect evidence of membrane disruption and discarded if such occurred. Below we will use the terms one-, two- and three- phase incubations to denote the conditions of treatment of the oocytes. *One-phase incubation*: Oocytes were bathed in a drop of 34 nM M-PEG-qdot in Ringer solution for 5–10 min and imaged. *Two-phase*

incubation: To determine the binding of M-PEG-qdots in the presence of putative competitors, we conducted experiments that involved initial incubation with the test competitor, followed by incubation in the presence of both test competitor and M-PEG-qdots. The test competitors investigated were: GABA (100 μ M to 5 mM); free muscimol (100 μ M to 5 mM); unconjugated qdots (34 nM); a conjugate consisting of PEG 3400 joined to muscimol ("PEG-muscimol") (500 μ M); and a conjugate that consisted of PEG 2000 joined to qdots ("PEG-qdots") (34 nM). The first phase of each experiment involved 15-min incubation with the test competitor at defined concentration. At the conclusion of this first-phase incubation, and after collection of an initial image when the test competitor was itself fluorescent (unconjugated qdots or PEG-qdots), the oocyte was removed from the surrounding medium, transferred to a drop (~35 μ l) of medium that contained the test competitor (at a concentration identical to that of the first incubation) along with 34 nM M-PEG-qdots, and maintained for 15 min in this latter medium (second-phase incubation). The oocyte was imaged at the conclusion of this second incubation. *Three-phase incubation:* Immediately following the two-phase protocol just described, the oocyte was removed from the medium containing GABA (100 μ M – 5 mM) plus 34 nM M-PEG-qdot, washed twice in Ringer for 5 min each, and then incubated for 5–10 min in 34 nM M-PEG-qdot alone (third-phase incubation), and then immediately imaged.

Fluorescence was measured using a confocal microscope (Leica model DM-IRE2 with 20X objective) with excitation at 476 nm. Fluorescence emission was detected over a wavelength interval (580–620 nm) that included the qdot emission peak (λ = 605 nm). Microscope settings relevant to excitation illumination and detection of fluorescence emission (gain and offset) were established at the beginning of experiments conducted on a given day, using either a human ρ 1 or perch ρ 1B- expressing oocyte bathed in 34 nM M-PEG-qdot, and maintained without change for the entire day's measurements. For optical clarity of both the fluorescence and bright-field images obtained from the (opaque) oocyte, the microscope was focused on the oocyte's equatorial plane. In all experiments, the pinhole was maintained at one airy unit to maximize resolution. On a given day, each set of experiments was performed on a single batch of oocytes and employed a single preparation of M-PEG-qdot in Ringer solution. Experiments involving single-step incubations were conducted both on oocytes expressing human ρ 1 and oocytes expressing perch ρ 1B GABA_C receptors; most two- and three-step protocols were performed on oocytes expressing human ρ 1 GABA_C receptors.

Image analysis

Each fluorescence image was analyzed using MetaMorph Offline version 6.3r0 software (Universal Imaging Corp., Downingtown, PA), as follows. *Oocyte surface membrane:* Under visual control, the cursor was used to trace the arc-like border of the oocyte as a series of 15–25 straight-line segments that spanned the entire field of view of the border, and included 450–750 pixels (i.e. data points). The cumulative length of this multi-segmented line was noted. Tabulated data for the "Border" region (see Results) indicate fluorescence intensity values for the pixels covered by this multi-segment line. *Surrounding medium:* Fluorescence of the medium bathing the oocyte was taken as a background to which the oocyte border fluorescence was referred. For determination of background fluorescence, the multi-segment line used to determine the border fluorescence was copied and replicated to cover a representative region in the medium (mean translation distance: 46 μ m; range: 34–75 μ m); the intensities of pixels covered by this segmented line were then obtained (tabulated data for the "Background" region in the Results). *Oocyte interior:* The fluorescence of the oocyte interior was in all cases comparable to or less than that of the external region and was not quantitatively analyzed.

The assessment of fluorescence intensity differences both within and between images required consideration of sample-to-sample variability for both the border and background characterization. To account for this variability we conducted analyses of variance (ANOVA).

This type of analysis allows for the evaluation of differential changes across images when, for example, the background intensities fluctuate. In addition, due to the punctate nature of the fluorescence, i.e., to the presence of “hot spots” of high fluorescence intensity, we anticipated the occurrence of non-normal distributions of pixel intensities across an image. To evaluate the distribution of signal intensities both within border and background regions of a given image and between borders and backgrounds across images we assessed the shape of the fluorescence intensity distributions using a chi-square (χ^2), a non-parametric test. In those cases for which there was a significant shift in the shape of the distribution, we have included the appropriate χ^2 statistics in the text. Throughout the Results text, differences in image intensities are evaluated by means of ANOVA and differences or shifts in the distribution of signal intensities are characterized by χ^2 . Because of the large sample size analyzed (450–750 pixel values per image), most comparison analyses yielded results with extremely low values ($p < 10^{-6}$), regardless of the differences between mean values. Accordingly, to evaluate the significance of the similarities or differences in data sets being compared, we determined values of eta-square (η^2), a parameter that characterizes the magnitude of the differences for each statistical comparison.⁴⁷ The value of η^2 ranges from 0 to 1, with $\eta^2 \leq 0.01$ conventionally classified as small, $\eta^2 \leq 0.06$ as moderate, and $\eta^2 \geq 0.14$ as large.^{47,48}

RESULTS

Binding of M-PEG-qdots

To assess the interaction between the synthesized M-PEG-qdot and GABA_C receptors, we tested whether we could visualize binding of the fluorescent compound at the surface of oocytes expressing either human $\rho 1$ or perch $\rho 1B$ GABA_C receptors. Fig. 4 shows results obtained with a one-phase incubation from GABA_C expressing and control oocytes. Fig. 4A shows results obtained with incubation of a human $\rho 1$ -expressing oocyte in the presence of 34 nM M-PEG-qdots. Here the fluorescence image of the oocyte was obtained after 5-min incubation in the M-PEG-qdot-containing medium. The image shows a thin halo of fluorescence at the oocyte surface, the intensity of which exceeded the surround fluorescence. The fluorescence image may be compared with the simultaneously obtained bright-field image, which illustrates the position and focus of the oocyte. A relatively sharp halo of fluorescence was observed with a perch $\rho 1B$ -expressing oocyte similarly treated with M-PEG-qdots in a single-phase incubation (Fig. 4B). Within a given group, results obtained with oocytes expressing either human $\rho 1$ or perch $\rho 1B$ GABA_C receptors, and incubated in the presence of M-PEG-qdots only, were taken as the positive control. By contrast with the results shown in panels A–B, only diffuse surround fluorescence was observed when an untreated (i.e., non-injected) oocyte that presumably lacked GABA_C receptors was incubated with M-PEG-qdots (Fig. 4C). Similarly, only diffuse surround fluorescence was observed with oocytes that received an injection of water rather than cRNA solution (not illustrated). Because diluting the M-PEG-qdot preparation in borate buffer into Ringer yields a solution with a pH of about 7.9, we assessed whether the elevated pH played a role in the binding, by lowering the pH of a 34 nM PEG-M-qdot solution to 7.2 with HCl, and testing its binding to GABA_C-expressing and non-expressing oocytes. Under these conditions, a fluorescence halo was observed for all tested GABA_C-expressing oocytes ($n=4$), but not for non-expressing oocytes ($n=3$) (data not shown).

The border of the oocyte described by Fig. 4A was analyzed for fluorescence intensity (see Experimental Section). For the human $\rho 1$ -expressing oocyte, this analysis yielded 67.31 ± 36.79 , as shown by the “Border” entry in Table 1, row 1. By comparison, fluorescence analysis of a representative multi-segment line in the surround medium, henceforth termed the background fluorescence, exhibited a significantly lower fluorescence intensity of 22.30 ± 21.18 (row 1, “Background”) (ANOVA: $F(1,580) = 696.26$, $p < 10^{-6}$, $\eta^2 = 0.54$). Border and background data obtained from the perch $\rho 1B$ -expressing oocyte of Fig. 4B similarly exhibited

a large border vs. background difference and large SDs (Table 1, row 2) ($F(1,479) = 814.51$, $p < 10^{-6}$, $\eta^2 = 0.63$). For the non-expressing oocyte of Fig. 4C, fluorescence intensity of the border did not exceed that of the background (Table 1, row 3). Furthermore, 2-way ANOVA showed that the relationship between border and background intensity depended on receptor expression, specifically showing a significant difference in border vs. background intensity for $\rho 1$ - and $\rho 1B$ -expressing oocytes but not for non-expressing oocytes ($F(2,1572) = 411.79$, $p < 10^{-6}$, $\eta^2 = 0.34$; where border and background measures are within-sample comparisons and $\rho 1$, $\rho 1B$, and non-expressing oocytes are between-sample comparisons). We interpret the relatively large SDs of the border data for the $\rho 1$ - and $\rho 1B$ -expressing oocytes to be due to non-homogeneous coverage of the oocyte surface membrane by the M-PEG-qdots (due, presumably, to non-uniform expression and/or clustering of GABA receptors on the cell membrane). The large SDs for the background data, and for the border data obtained from the non-expressing oocyte, are interpreted as due to the punctuate nature of surround fluorescence of the qdot suspension, together with a possible aggregation of the M-PEG-qdots. Further insight into the characteristics of fluorescence properties described by rows 1–3 in Table 1 comes from considering the distribution of fluorescence intensity of the pixels themselves. Information on the shapes of these distributions is shown by the histograms of Fig. 4, which illustrate distributions of pixels for the border and background regions of the corresponding Fig. 4 fluorescence image.

Rows 4–6 of Table 1 show aggregate results obtained for border and background fluorescence among groups of oocytes. These data were obtained from measurements conducted on multiple replicates (human $\rho 1$; positive control: $n = 11$; perch $\rho 1B$ positive control: $n = 4$; negative control: $n = 14$) on different experiment days. Comparison by 2-way mixed-design ANOVA of the border versus background fluorescence for the human $\rho 1$ - and perch $\rho 1B$ -expressing oocytes, relative to non-expressing oocytes, indicated a significant difference in fluorescence intensity ($F(2,15507) = 4285.13$, $p < 10^{-6}$, $\eta^2 = 0.37$). Specifically, the respective values for border and background regions were 88.84 ± 64.84 and 31.60 ± 35.50 for human $\rho 1$ -expressing oocytes; 109.58 ± 58.42 and 18.54 ± 16.47 for perch $\rho 1B$ -expressing oocytes; and 15.14 ± 22.35 and 16.78 ± 22.17 for non-expressing oocytes. This two-way ANOVA demonstrates a significant difference between border and background intensities for both the human $\rho 1$ and perch $\rho 1B$, whereas there is no difference between border and background intensities for the non-expressing oocytes. Near-maximum fluorescence intensity was typically reached early in the period of incubation. That is, fluorescence intensities measured after 2–3 and 5 min of exposure of GABA_C-expressing oocytes to M-PEG-qdots (data not shown) were comparable with intensities measured after 10-min incubation (Figs. 4 and 5, and Table 1).

For the aggregate data obtained from multiple replicates (groups identified above), there was a significant difference in both the intensity and the shape of the distribution of values between border relative to background values for human $\rho 1$ -expressing oocytes ($F(1,6376) = 6050.72$, $p < 10^{-6}$, $\eta^2 = 0.49$; $\chi^2(11929) = 12567.6$, $p = 2.4 \times 10^{-5}$). Similarly, for perch $\rho 1B$ -expressing oocytes, there was a significant difference in both shape (Fig. 5) and intensity of the distribution for border vs. background regions ($F(1,1685) = 4028.97$, $p < 10^{-6}$, $\eta^2 = 0.71$; $\chi^2(7420) = 7751.8$, $p = 0.004$). Border vs. background data obtained from non-expressing oocytes also showed a significant difference in the distribution of intensity values, but, by contrast with the results obtained from human $\rho 1$ and perch $\rho 1B$ -expressing oocytes, showed no difference in distribution shape ($F(1,7446) = 27.93$, $p < 10^{-6}$, $\eta^2 = 0.004$; $\chi^2(3944) = 4086.5$, $p = 0.056$).

The removal of M-PEG-qdots from GABA_C-expressing oocytes was investigated in wash-out experiments. Following treatment for 5–10 min with 34 nM M-PEG-qdots, the oocyte was immersed for 2–5 s in ligand-free medium and then transferred to a static bath of ligand-free medium (Ringer solution) for further incubation. Fig. 6 shows results obtained in experiments of this type on human $\rho 1$ and perch $\rho 1B$ GABA_C receptor-expressing oocytes. In each

experiment, normalized fluorescence intensity F/F_0 of the border region was determined at the conclusion of the incubation with M-PEG-qdots ($t=0$), and defined times following transfer of the oocyte into the initially ligand-free medium. The data obtained from human $\rho 1$ and perch $\rho 1B$ GABA_C receptor-expressing oocytes indicated that a 50% decrease in normalized fluorescence intensity required incubation for roughly 10–15 min in this latter medium.

Competition with M-PEG-qdot binding

Two-phase incubations—To test whether known GABA_C agonists and other test agents can compete with M-PEG-qdot for binding, we conducted experiments involving two-phase incubation of the oocyte. In the first of these two phases, the oocyte was bathed for 15–20 min in medium containing the test agent at defined concentration. At the conclusion of this first “pre-treatment” incubation period, the oocyte was immediately transferred to medium containing both the same concentration of test agent as well as 34 nM M-PEG-qdots, and further incubated for 10–15 min. The fluorescence image obtained at the conclusion of this second incubation was analyzed in relation to the image obtained from a positive control oocyte incubated only with 34 nM m-PEG-qdots. Figs. 7A–B show, respectively, results obtained from a positive control oocyte (**A**) and those obtained from an oocyte subjected to 100 μ M GABA treatment through the two-phase protocol just described (**B**). By comparison with the image obtained from the positive control, that obtained with 100 μ M GABA as test agent exhibited relatively little border fluorescence. The border intensity of this GABA-treated oocyte (Table 1, row 7) was significantly lower than that of the positive control run on the same experiment day (83.34 ± 64.82 , not shown in Table 1), and border distribution was significantly shifted from that of the control ($F(1,1067) = 485.26$, $p < 10^{-6}$, $\eta^2 = 0.31$; $\chi^2(16770) = 17217.67$, $p = 0.008$). Two-phase incubation with either 500 μ M GABA or 5 mM GABA also significantly reduced border intensity (for 500 μ M GABA, $F(1, 1123) = 784.54$, $p < 10^{-6}$, $\eta^2 = 0.41$; and for 5 mM GABA, $F(1, 1052) = 639.06$, $p < 10^{-6}$, $\eta^2 = 0.38$). Furthermore, significant reductions in border intensity and shifts in distribution shape were produced by two-phase treatment with 100 μ M muscimol (Fig. 7C; Table 1, row 8), with 500 μ M and with 5 mM muscimol (data obtained under the latter two conditions not illustrated). Specifically, for 100 μ M muscimol, $F(1,1057) = 573.95$, $p < 10^{-6}$, $\eta^2 = 0.35$; $\chi^2(13056) = 13375.85$, $p = 0.025$; for 500 μ M muscimol, $F(1,1235) = 922.75$, $p < 10^{-6}$, $\eta^2 = 0.43$; and for 5 mM muscimol, $F(1,1158) = 843.37$, $p < 10^{-6}$, $\eta^2 = 0.42$. In addition, two-phase incubation with 500 μ M M-PEG, a structure lacking the qdot, significantly reduced border intensity (Fig. 7D; Table 1, row 9) ($F(1,1157) = 733.77$, $p < 10^{-6}$, $\eta^2 = 0.39$).

Two-phase experiments also were conducted with two qdot-based (and thus, fluorescent) structures in which the muscimol moiety was absent. In these experiments, images were obtained at the end of the first-phase incubation (treatment with the test competitor alone) as well as at the end of the second-phase incubation with both test competitor and 34 nM M-PEG-qdots. Fig. 8A shows results obtained from the positive control preparation (human $\rho 1$ -expressing oocyte incubated with 34 nM M-PEG-qdots) investigated on the experiment day under consideration. Fig. 8B–C and Table 1, rows 10–11 show results obtained from a human $\rho 1$ GABA_C-expressing oocyte treated with unconjugated qdots (a structure lacking both poly (ethylene glycol) and muscimol) during the two phases of incubation. To compare the post-first-phase image (which itself exhibited fluorescence; Fig. 8B and Table 1, row 10) and post-second phase image (8C and Table 1, row 11), we used two-way ANOVA with border and background measurements for within-sample comparison and incubation phase for between-sample comparison. This allowed evaluation not only of the relationship between post-first-phase and post-second-phase border intensity, but also of the ratio of border vs. background intensity across the two incubation phases. The results ($F(1,1142) = 377.85$, $p < 10^{-6}$, $\eta^2 = 0.25$) indicated that the change in border intensity between post-phase 1 (15.79 ± 23.18) and post-phase 2 (89.31 ± 58.62) was significant (as was the change in background intensity between

post-phase 1 and post-phase 2; 13.13 ± 18.17 and 39.99 ± 23.80 , respectively). The ratio of border to background intensity also increased significantly between post-phase 1 and post-phase 2.

Generally similar results were obtained when human $\rho 1$ GABA_C-expressing oocytes were treated with PEG-qdots, i.e., with qdots conjugated to PEG 2000 that lacked an (aminohexanoyl) muscimol terminating group (Figs. 8D–E and Table 1, rows 12–13). ANOVA of the post-phase 1 and post-phase 2 fluorescence data yielded ($F(1,1101) = 1324.40$, $p < 10^{-6}$, $\eta^2 = 0.55$; $\chi^2(3848) = 4138.6$, $p = 0.001$), i.e., indicated a significant increase in border intensity upon phase 2 incubation with M-PEG-qdots. As observed with unconjugated qdots, the post-phase 1 border intensity with PEG-qdots was significantly lower than the border intensity of the positive control. However, by contrast with results obtained with unconjugated qdots, background fluorescence intensity obtained with PEG-qdots was small by comparison with that of the positive control. We interpret the low overall (i.e., both border and background) fluorescence obtained with PEG-qdots as due at least in part to a reduction in fluorescence quenching associated with the relatively high ligand loading of the PEG-qdot preparation (see Experimental Section).

The results of these experiments with unconjugated qdots and PEG-qdots argue against a non-specific binding of the qdot moiety to GABA_C receptors or other components of the oocyte surface membrane as the basis of the binding activity described in the experiments of Figs. 4, 5 and 7.

Three-phase incubations—Fig. 7, and the accompanying tabular and statistical data described above, indicate that agonists such as GABA at $\geq 100 \mu\text{M}$ concentration, when co-incubated with 34 nM M-PEG-qdots, significantly reduce the binding of M-PEG-qdots as determined at the end of the (second-phase) incubation. To investigate whether this competition with M-PEG-qdot binding is reversible, we employed a three-phase protocol in which the second incubation was followed by washing of the oocyte and a further incubation with 34 nM M-PEG-qdots only. Fig. 9A shows the result obtained from the positive control $\rho 1$ -expressing oocyte investigated on the day of the experiment to be described. Figs. 9B–C show results obtained from an oocyte subjected to a three-phase incubation involving treatment with $100 \mu\text{M}$ GABA. The image B, which was obtained at the end of the second-phase incubation ($100 \mu\text{M}$ GABA plus 34 nM M-PEG-qdot), may be compared with that of C obtained at the end of the third-phase incubation with 34 nM PEG-qdot alone. Two-way ANOVA of the Fig. 9B–C data, using border and background measurements as within-sample measurements and condition/treatment as a between-sample measurement, indicated a significant increase in ratio of border to background intensity between the end of phase 2 and the end of phase 3 (Table 1, rows 14–15; 22.41 ± 32.71 vs. 119.51 ± 84.47) ($F(1,1229) = 679.95$, $p < 10^{-6}$, $\eta^2 = 0.36$). Furthermore, the border intensity at the conclusion of phase 3 was higher than that of the positive control (71.80 ± 77.62) ($F(1,1100) = 100.22$, $p < 10^{-6}$, $\eta^2 = 0.08$), indicating essentially complete recovery of M-PEG-qdot binding. The relationship among the fluorescence data obtained at the end of phase 2 (Fig. 9B), at the end of phase 3 (Fig. 9C), and from the positive control (Fig. 9A) is further described by the border and background distributions accompanying each image. For the border region, the distribution exhibited at the end of phase 3 barely reached the criterion for a significant difference with respect to the positive control. Furthermore, the border distribution exhibited at the end of phase 2 did not differ significantly from that exhibited at the end of phase 3; it did, however, differ markedly from that exhibited by the positive control ($\chi^2(21922) = 22373.75$, $p = 0.016$).

Results similar to those of the Fig. 9 experiment were obtained in three-phase experiments involving treatment with $500 \mu\text{M}$ GABA or with 5 mM GABA (not illustrated). With $500 \mu\text{M}$ GABA, the ratio of border to background intensity obtained at the end of phase 3 ($123.85 \pm$

84.67), i.e., in the presence of 34 nM M-PEG-qdots alone, differed significantly from those obtained at the end of phase 2 (44.43 ± 48.26), i.e., in the presence of both 500 μ M GABA and 34 nM M-PEG-qdots (2-way ANOVA, $F(1,1040) = 350.73$, $p < 10^{-6}$, $\eta^2 = 0.25$). With 5 mM GABA, there was a significant shift in border values between the end of phase 2 (30.32 ± 28.51) and the end of phase 3 (54.26 ± 37.35) (2-way ANOVA, $F(1,1076) = 138.01$, $p < 10^{-6}$, $\eta^2 = 0.11$).

DISCUSSION

The present study has examined the interaction of GABA_C receptors with a conjugate synthesized from CdSe qdots, with the receptor agonist muscimol tethered distally in multiple copies to the qdot by a PEG linker. The primary finding is that the qdot-tethered form of muscimol investigated here exhibits specific binding to expressed GABA_C receptors, and that this binding activity depends on the presence of muscimol in the conjugate. Three types of evidence support this view. First, the compound binds selectively to oocytes expressing GABA_C receptors. Second, results obtained from the two-phase experiments indicate an inhibition of M-PEG-qdot binding by GABA (>100 μ M), muscimol (>100 μ M), and M-PEG (500 μ M) (i.e., a competition, by these agents, with M-PEG-qdot binding); an absence of substantial binding activity by structures lacking muscimol (unconjugated qdots and PEG-qdots); and little if any competition by the muscimol-lacking structures with the binding of M-PEG-qdots. Third, results of the three-phase experiments indicate that the removal of competing GABA restores the muscimol-PEG-qdot binding ability of the GABA_C receptors. To our knowledge, the present study is the first to provide evidence for binding activity, at the ligand-binding pocket of a ligand-gated ion channel, of a receptor agonist covalently joined to a qdot.

The approach used here builds on that employed by Rosenthal *et al.*,²⁸ who synthesized a conjugate consisting of AMP-coated CdSe qdots covalently joined to serotonin through a short linker arm. In labeling, transport, and electrophysiological experiments employing this conjugate, Rosenthal *et al.*,²⁸ found that the conjugate exhibits specific binding activity at human and drosophila serotonin transporters expressed in HeLa and HEK-293 cells, and inhibits transport of serotonin in HeLa cells expressing serotonin transporters. The conjugate lacked electrophysiological activity on *Xenopus* oocytes expressing the serotonin receptor but, on oocytes expressing the serotonin transporter, exhibited activity similar to that of antagonists. Other investigators have used qdots coupled covalently to various other functional groups to target receptors *in vitro* and *in vivo*. Examples include the use of transferrin-qdot conjugates that underwent receptor-mediated endocytosis;² of lung-targeting peptide-qdot conjugates injected *in vivo* into the peripheral circulation;¹⁹ of EGF-qdots to target receptor tyrosine kinase and quantify EOF binding and internalization;²⁰ and of peptide-qdot conjugates to image the angiotensin receptor.^{49,50} In addition, streptavidin-coated qdots conjugated noncovalently with biotinylated biomolecules (e.g. biotinylated bombesin or angiotensin n) have recently been used to target G- protein-coupled receptors,⁵¹ glial cells,⁵² biomolecules such as single kinesins,⁵³ and specific proteins in living cells.⁵⁴ Other investigations have employed biotinylated antibodies or peptides conjugated with streptavidin-coated qdots as targeting agents.^{23,55–57}

The design of the qdot-conjugated structure used in the present experiments was motivated by a recent study indicating that a chain-derivatized form of muscimol containing a sterically bulky biotin group ("muscimol-biotin") exhibits agonist activity at GABA_C and GABA_A receptors expressed in *Xenopus* oocytes.^{46,58} Muscimol-biotin differs from the present M-PEG-qdot in that it contains muscimol conjugated to biotin through a short hydrocarbon (aminohexanoyl) linker, rather than to a qdot through a long PEG (plus aminohexanoyl) linker. Available data furthermore indicate an interaction between GABA_A receptors in retinal

neurons and muscimol conjugated to the bulky fluorophore BODIPY[®];⁵⁹ electrophysiological activity of muscimol-BODIPY[®] at GABA_A and GABA_C receptors;⁴⁶ and high-affinity interaction between GABA_A receptors of rat hippocampal neurons and muscimol conjugated to Alexa Fluor 532.⁶⁰ In light of the evident binding activity of the present qdot-based structure and the previous studies just summarized, we conclude that muscimol, when conjugated to a sterically bulky component (e.g., qdot or biotin) through a linker that avoids steric clash of this bulky component with the receptor, retains receptor-binding activity. The present results specifically demonstrate the ability of the long-chain PEG linker used here to permit binding of the muscimol moiety at the GABA_C ligand-binding site, i.e., to achieve sufficient distance of the qdot platform from the GABA_C binding pocket.

A striking property of the conjugate is the strength of its interaction with oocyte-expressed GABA_C receptors. This interaction may depend in part on the presence of the PEG-qdot structure in the conjugate. As indicated by the wash-out experiments of Fig. 6, half-reduction of the fluorescence signal due to membrane-bound M-PEG-qdots requires approximately 10–15 min of incubation following transfer into ligand-free medium. Interestingly, electrophysiological data obtained with muscimol-biotin and muscimol-BODIPY at oocyte-expressed GABA_C receptors indicate that the exponential time constant for recovery of the agonist response to these compounds substantially exceeds that for muscimol itself (about 15 s for muscimol-biotin and muscimol-BODIPY, vs. about 4 s for muscimol). As discussed by Vu *et al.*,⁴⁶ the relatively long recovery time course of the electrophysiological response to muscimol-biotin and muscimol-BODIPY may derive to some extent from an (as yet undetermined) interaction of the terminating biotin or BODIPY group conjugated to muscimol in these structures. Conceivably, the evident slowness of wash-out of M-PEG-qdots observed in the present fluorescence experiments could reflect a long-persisting interaction of the tethering qdot with the receptor. If this is the case, such a qdot/receptor interaction must depend on conjugation of the qdot with muscimol, as neither unconjugated qdots nor PEGylated qdots exhibit substantial binding (Fig. 8 and Table 1). Previous studies have shown that quantum dots coated in an amphiphilic polymer shell bind nonspecifically to mammalian cell membranes. However, when these quantum dots are PEGylated, this nonspecific binding is significantly reduced.^{29,49}

A further factor that may play a role in the affinity of the M-PEG-qdot for the GABA_C receptor concerns its multivalency, since the conjugate contains an average of approximately 150–200 PEG-muscimol ligands coupled to each qdot. This multivalency can be expected to increase the effective concentration of muscimol locally available for interaction with neighboring GABA_C ligand binding sites when the M-PEG-qdot is bound through at least one muscimol's binding. That is, the high avidity of the M-PEG-qdot structure is expected to promote retention of the M-PEG-qdot at the oocyte surface membrane, and could contribute also to the evident time course of M-PEG-qdot wash-out (Fig. 6). Interestingly, Lester *et al.*⁶¹ found that a bivalent form of acetylcholine exhibits high binding activity to the acetylcholine receptor, a ligand-gated ion channel of structure generally similar to that of GABA_A and GABA_C receptors. Studies by Kula *et al.*⁶² and by Lin and Licht⁶³ have shown that other Y-shaped or bivalent ligands also exhibit activity at acetylcholine receptors. These considerations, together with the presence of multiple ligand-binding sites on the pentameric receptor⁶⁴ and evidence for the cooperativity of ligand binding to GABA receptors⁶⁵ are consistent with occurrence of a specific affinity of M-PEG-qdots for the receptor.

In conclusion, the present findings describe the specific recognition, by a neurotransmitter membrane receptor, of a multivalent agonist-containing structure in which the multiple copies of receptor ligand are tethered to a sterically bulky distal component (here, the qdot) through a suitably long linker. The evident binding activity of this type of conjugate in the present GABA_C system raises the possibility that a sterically bulky structure of nanometer or larger

scale positioned at or near the receptor's extracellular surface could control receptor activity by governing the access, to receptor binding pockets, of ligands tethered to the external structure. The present findings thus encourage the investigation of such structures for possible applications in the control or modulation of neural signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Ms. Ruth Zelkha for her expert assistance with cell imaging and fluorescence analysis, Mr. Niraj J. Muni for oocyte preparation, Dr. Robert F. Standaert for helpful discussions, and Quantum Dot Corporation for providing the AMP®-coated quantum dots used in this study. Supported by NIH grants EY13693, EY05494, EY01792, EB003728, EM72048 and MH075791; by a grant from the University of Illinois Inter-campus Research Initiative in Biotechnology Program; by a Macular Degeneration Research grant from the American Health Assistance Foundation; and by an unrestricted departmental award from Research to Prevent Blindness (New York, NY). D.R.P. is a Senior Scientific Investigator of Research to Prevent Blindness. Preliminary results were presented at the 2005 and 2006 Annual meetings of the Association for Research in Vision and Ophthalmology.

References

1. Bruchez M Jr, Moronne M, Gin P, Weiss S, Alivisatos AP. Science 1998;281:2013–2016. [PubMed: 9748157]
2. Chan WCW, Nie S. Science 1998;281:2016–2018. [PubMed: 9748158]
3. Alivisatos AP. J Phys Chem 1996;100:13226–13239.
4. Murray CB, Norris DJ, Bawendi MG. J Am Chem Soc 1993;115:8706–8715.
5. Hines MA, Guyot-Sionnest P. J Phys Chem 1996;100:468–471.
6. Alivisatos P. Nature Biotechnol 2004;22:47–52. [PubMed: 14704706]
7. Kim S, Yong TL, Soltesz EG, De Grand AM, Lee J, Nakayama A, Parker JA, Mihaljevic T, Laurence RG, Dor DM, Cohn LH, Bawendi MG, Frangioni JV. Nature Biotechnol 2004;22:93–97. [PubMed: 14661026]
8. Dubertret B, Skourides P, Norris DJ, Noireaux V, Bivantalou AH, Libchar A. Science 2002;298:1759–1762. [PubMed: 12459582]
9. Gerion D, Pinaud F, Williams SC, Parak WJ, Zanchet D, Weiss S, Alivisatos PA. J Phys Chem 2001;105:8861–8871.
10. Jovin TM. Nature Biotechnol 2003;21:23–33.
11. Gao X, Cui Y, Levenson RM, Chung LWK, Nie S. Nature Biotechnol 2004;22:969–976. [PubMed: 15258594]
12. Wu X, Liu H, Liu J, Haley KN, Treadway JA, Larson JP, Ge N, Peale F, Bruchez M. Nature Biotechnol 2003;21:41–46. [PubMed: 12459735]
13. Chunyang Z, Hui M, Yao D, Lei J, Dieyan C, Shuming N. Analyst 2000;125:1029–1031.
14. Hoshino A, Hanaki K, Suzuki K, Yamamoto K. Biochem Biophys Res Comm 2004;314:46–53. [PubMed: 14715244]
15. Bäuml M, Stamou D, Segura J, Hovius J, Vogel H. Langmuir 2004;20:3828–3831. [PubMed: 15969364]
16. Michalet X, Fabien P, Lacoste TD, Dahan M, Bruchez MP, Alivisatos AP, Weiss S. Single Mol 2001;4:261–276.
17. Goldman ER, Balighian ED, Mattoussu H, Kuno KM, Mauro JM, Tran PT, Anderson GP. J Am Chem Soc 2002;124:6378–6382. [PubMed: 12033868]
18. Minet O, Dressler C, Beuthan J. J Fluoresc 2004;14:241–247. [PubMed: 15615205]
19. Åkerman ME, Chan WCW, Laakkonen P, Bhatia SN, Ruoslahti E. Proc Natl Acad Sci USA 2002;99:12617–12621. [PubMed: 12235356]

20. Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. *Nature Biotechnol* 2004;22:198–203. [PubMed: 14704683]
21. Watson A, Wu X, Bruchez M. *Bioimaging* 2003;34:296–303.
22. Goldman ER, Clapp AR, Anderson GP, Uyeda HT, Mauro JM, Medintz IL, Mattoussi H. *Anal Chem* 2004;76:684–688. [PubMed: 14750863]
23. Dahan M, Lévi S, Luccardini C, Rostaing P, Riveau B, Triller A. *Science* 2003;302:442–445. [PubMed: 14564008]
24. Tomlinson ID, Burton JN, Mason JN, Blakely R, Rosenthal SJ. *Tetrahedron* 2003;59:8035–8047.
25. Tomlinson ID, Grey JL, Rosenthal SJ. *Molecules* 2002;7:777–790.
26. Tomlinson ID, Mason JN, Blakely RD, Rosenthal SJ. *Bioorg Med Chem Lett* 2005;15:5307–5310. [PubMed: 16183285]
27. Tomlinson ID, Kippeny T, Siddiqui N, Rosenthal SJ. *J Chem Res* 2002;5:202–203.
28. Rosenthal SJ, Tomlinson ID, Adkins EM, Schroter S, Adams S, Swafford L, McBride J, Wang Y, De Felice LJ, Blakely RD. *J Am Chem Soc* 2002;124:4586–4594. [PubMed: 11971705]
29. Bentzen EL, Tomlinson ID, Mason J, Gresch P, Wright D, Sanders-Busch E, Blakely R, Rosenthal SJ. *Bioconj Chem* 2005;16:1488–1494.
30. Qian H, Dowling JE. *J Neurosci* 1994;14:4299–4307. [PubMed: 8027780]
31. Enz R, Brandstätter JH, Wässle H, Bormann J. *J Neurosci* 1996;16:4479–4490. [PubMed: 8699258]
32. Euler T, Wässle H. *J Neurophysiol* 1998;79:1384–1395. [PubMed: 9497419]
33. Lukasiewicz PD, Shields CR. *J Neurophysiol* 1998;79:3157–3167. [PubMed: 9636116]
34. Shen W, Slaughter MM. *J Physiol* 2001;530:55–67. [PubMed: 11136858]
35. Gibbs ME, Johnston GAR. *Neuroscience* 2005;13:567–576. [PubMed: 15730863]
36. Lukasiewicz PD. *Prog Brain Res* 2005;147:205–218. [PubMed: 15581708]
37. Zhang D, Pan ZH, Zhang X, Brideau AD, Lipton SA. *Proc Natl Acad Sci USA* 1995;92:11756–11760. [PubMed: 8524843]
38. Milligan CJ, Buckley NJ, Garret M, Deuchars J, Deuchars SA. *J Neurosci* 2004;24:7241–7250. [PubMed: 15317850]
39. Pan Y, Qian HJ. *Neurochem* 2005;94:482–490.
40. Qian H, Dowling JE, Ripps H. *J Neurobiol* 1998;37:305–320. [PubMed: 9805275]
41. Chebib M. *Clin Exper Pharmacol Physiol* 2004;31:800–804. [PubMed: 15566397]
42. Sedelnikova A, Smith CD, Zakharkin SO, Davis D, Weiss DS, Chang Y. *J Biol Chem* 2005;280:1535–1542. [PubMed: 15548535]
43. Frey M, Jäger V. *Synthesis* 1985:1100–1104.
44. Doughty MB, Chaurasia CS, Li K. *J Med Chem* 1993;36:272–279. [PubMed: 8093737]
45. Kippeny T, Swafford LA, Rosenthal SJ. *J Chem Ed* 2002;79:1094–1100.
46. Vu TQ, Chowdhury S, Muni NJ, Qian H, Standaert RR, Pepperberg DR. *Biomaterials* 2005;26:1895–1903. [PubMed: 15576163]
47. Cohen, J. *Statistical power analysis for the behavioral sciences*. Hillsdale, NJ, USA: Lawrence Erlbaum Associates, Inc; 1977.
48. Stevens, J. *Applied Multivariate Statistics for the Social Sciences*. 3. Erlbaum; Hillsdale, NJ: p. 1771996
49. Tomlinson ID, Mason JN, Blakely RD, Rosenthal SJ. *Methods Mol Biol* 2005;303:51–60. [PubMed: 15923674]
50. Mason JN, Farmer H, Tomlinson ID, Schwartz JW, Savchenko V, DeFelice LJ, Rosenthal SJ, Blakely RD. *J Neurosci Meth* 2005;143:3–25.
51. Young SH, Rozengurt E. *Am J Physiol Cell Physiol* 2006;290:C728–C732. [PubMed: 16236822]
52. Pathak S, Cao E, Davidson MC, Jin S, Silva GA. *J Neurosci* 2006;26:1893–1895. [PubMed: 16481420]
53. Seitz A, Surrey T. *EMBO J* 2006;25:267–277. [PubMed: 16407972]
54. Howarth M, Takao K, Hayashi Y, Ting AY. *Proc Natl Acad Sci USA* 2005;102:7583–7588. [PubMed: 15897449]

55. Mason JN, Tomlinson ID, Rosenthal SJ, Blakely RD. *Methods Mol Biol* 2005;303:35–50. [PubMed: 15923673]
56. Vu TQ, Maddipati R, Blute TA, Nehilla BJ, Nusblat L, Desai TA. *Nano Letters* 2005;5:603–607. [PubMed: 15826094]
57. Yang L, Li Y. *Analyst* 2006 2006;131:394–01.
58. Nehilla BJ, Popat KC, Vu TQ, Chowdhury S, Standaert RF, Pepperberg DR, Desai TA. *Biotechnol Bioeng* 2004;87:669–674. [PubMed: 15352065]
59. Wang H, Standifer KM, Sherry DM. *Visual Neurosci* 2000;17:11–21.
60. Meissner O, Häberlein H. *Biochemistry* 2003;42:1667–1672. [PubMed: 12578381]
61. Lester HA, Chabala LD, Gurney AM, Sheridan RE. *Soc Gen Physiol Ser* 1986;40:447–462. [PubMed: 2424099]
62. Kula, MA.; Dunn, SMJ.; Dryden, WF. 2004 Annual Meeting of the Society for Neuroscience. 2004. Program Number 624.20 (abstr.)
63. Lin, WC.; Licht, S. 2006 Annual Meeting of the Biophysical Society. 2006. Program Number 56 (abstr.)
64. Chang Y, Weiss DS. *Nature Neurosci* 1999;2:219–225. [PubMed: 10195213]
65. Enz R, Brandstatter JH, Wassle H, Bormann J. *J Neurosci* 1996;16:4479–4490. [PubMed: 8699258]

Figure 1. Structure of the investigated muscimol-PEG-qdot (M-PEG-qdot) preparation. The sphere represents the AMP-coated CdSe/ZnS qdot.

Figure 2.

Synthetic schemes for compounds that were conjugated to qdots. **A:** Route used for synthesis of muscimol-containing compound **4**. (i) Pyridine, room temperature, 18 hr; (ii) trifluoroacetic acid, room temperature, 1 hr; (iii) t-Butoxycarbonyl amine-PEG-activated acid, pyridine, room temperature, 18 hr; (iv) trifluoroacetic acid, methylene chloride, room temperature, 1 hr. Here and in panel **B**, the value of the subscript *n* (denoting the number of ethylene glycol units in PEG 3400) is ~78. **B:** Route for synthesis of compound **7**. (i) Potassium hydroxide, para-toluene sulfonyl chloride, methylene chloride, 0 °C, 6 hours; (ii) Potassium phthalimide, acetonitrile, reflux, 18 hr; (iii) (a) Ethanol, hydrazine hydrate, room temperature, 18 hr; (b) Methylene chloride, room temperature, 18 hr.

Figure 3.

A: UV-visible spectrum of M-PEG-qdots. **B:** Agarose gel electrophoresis (1% agarose gel, TAE buffer; 80 mV potential difference) of AMP-coated qdots vs. muscimol-conjugated qdots. Lane 1: PEG2000-qdots. Lane 2: M-PEG-qdots. Lane 3: AMP-coated qdots.

Figure 4.

Representative single-phase experiments involving incubation of the oocyte with 34 nM M-PEG-qdots. **A–B:** Results obtained with oocytes expressing human ρ (A) and perch ρ IB (B) GABAc receptors. **C:** Results obtained with a non-injected oocyte. Within each panel (i.e., within each row of the figure) are shown a fluorescence image obtained from a single representative oocyte after a 10-min. incubation with the M-PEG-qdots, and the corresponding bright-field image of the (opaque) oocyte in the plane of focus. Each panel also indicates the distributions of fluorescence intensity for the border and background regions determined from the illustrated fluorescence image. Here and in subsequent Figures, distributions of border and background intensities are plotted with fixed bin widths, each spanning 15 gray scale units.

Figure 5.

Aggregate data for distributions of border and background intensity obtained in single-phase experiments on human $\rho 1$ -expressing oocytes (A) (n=11), perch $\rho 1B$ -expressing oocytes (B) (n=4) and non-expressing oocytes (C) (n=14).

Figure 6.

Wash-out of M-PEG-qdots from the border region of human $\rho 1$ -expressing and perch $\rho 1B$ -expressing oocytes ($n=4$ and $n=2$, respectively). In each experiment, fluorescence intensity of the border and background regions was determined following incubation with 34 nM M-PEG-qdots, rapid washing, and subsequent transfer into ligand-free medium (see text). The net difference in fluorescence intensity (border minus background) obtained at a given time was normalized to that obtained at the conclusion of incubation with the M-PEG-qdots, to yield the normalized fluorescence intensity F/F_0 . Filled circles show data obtained from human $\rho 1$ -expressing oocytes (1–4 determinations) as a function of time following transfer of the oocyte into the ligand-free medium. For multiple determinations, the data indicate the mean $\pm$ SD. Open circles show data obtained from perch $\rho 1B$ -expressing oocytes (1–2 determinations).

Figure 7.

Two-phase experiments in oocytes expressing human $\rho 1$ GABA_C receptors. **A:** Positive control for this series of observations (oocyte expressing human $\rho 1$, imaged after a 5-min incubation in 34 nM M-PEG-qdots). **B:** Two-phase incubation involving (1) 100 μ M GABA, and (2) 34 nM M-PEG-qdots in buffer supplemented with 100 μ M GABA. **C:** two-phase incubation involving (1) 100 μ M muscimol, and (2) 34 nM M-PEG-qdots in buffer supplemented with 100 μ M muscimol. **D:** Two-phase incubation involving (1) 500 μ M PEG-M, and (2) 34 nM M-PEG-qdots in buffer supplemented with 500 μ M PEG-M.

Figure 8.

Two-phase experiments with qdot-containing structures and oocytes expressing human $\rho 1$ GABA_C receptors. **A:** Positive control for this series of observations (oocyte expressing human $\rho 1$, imaged after a 5-min incubation in 34 nM M-PEG-qdot). **B–C:** oocyte after two-phase incubation involving (1) 34 nM unconjugated qdot (**B**), and (2) 34 nM M-PEG-qdot in buffer supplemented with 34 nM unconjugated qdot (**C**). **D–E:** oocyte after two-phase incubation involving (1) 34 nM PEG-qdot (**D**), and (2) 34 nM M-PEG-qdot in buffer supplemented with 34 nM PEG-qdot (**E**).

Figure 9.

Three-phase experiment involving competition with 100. μM GABA. **A:** Results obtained from a positive control oocyte (single-phase incubation with 34 nM M-PEG-qdot) examined on the same day as that of the experiments shown in B and C. **B–C:** Fluorescence images obtained with three-phase incubation, and with 100 μM GABA as a test competitor. Data obtained from a single oocyte following the second-phase incubation (medium containing 100 μM GABA and 34 nM M-PEG-qdots) (**B**), and after the third-phase incubation (medium containing only 34 nM M-PEG-qdots) (**C**). Distributions describe fluorescence intensities for border and background regions of the corresponding fluorescence images.

Fluorescence intensities of oocyte surface membrane (border) and surrounding regions (background).

Row	Figure	GABA _A Receptor	Test agent(s)	Border (mean ± SD) ^A	Background (mean ± SD) ^A
1	Fig. 4A	ρ	M-PEG-qdot	67.31 ± 36.79	22.30 ± 21.18
2	Fig. 4B	ρ1B	M-PEG-qdot	62.34 ± 38.56	9.65 ± 14.67
3	Fig. 4C	---	M-PEG-qdot	10.16 ± 18.17	18.56 ± 19.95
4	---	ρ	M-PEG-qdot (n=11) ^B	88.84 ± 64.84	31.60 ± 35.50
5	---	ρ1B	M-PEG-qdot * (n=4) ^B	109.58 ± 58.42	18.54 ± 16.47
6	---	---	M-PEG-qdot * (n=14) ^B	15.14 ± 22.35	16.78 ± 22.17
7	Fig. 7B	ρ1	(1) GABA (100 μM), (2) M-PEG-qdot plus 100 μM GABA	16.10 ± 25.54	4.58 ± 8.91
8	Fig. 7C	ρ1	(1) Muscimol (100 μM), (2) M-PEG-qdot plus 100 μM muscimol	12.16 ± 17.47	3.69 ± 8.31
9	Fig. 7D	ρ1	(1) M-PEG (500 μM), (2) M-PEG-qdot plus 500 μM M-PEG	32.05 ± 31.26	4.91 ± 10.27
10	Fig. 8B	ρ1 ^C	unconjugated qdot (34 nM)	16.29 ± 24.19	13.21 ± 18.16
11	Fig. 8C	Same ρ1 (10) ^C	(1) unconjugated qdot (34 nM), (2) M-PEG-qdot (34 nM) plus 34 nM unconjugated qdot	89.94 ± 58.07	30.71 ± 23.54
12	Fig. 8D	ρ1 ^C	PEG-qdot (34 nM)	1.52 ± 5.77	1.80 ± 6.39
13	Fig. 8E	Same ρ1 (12) ^C	(1) PEG-qdot (34 nM), (2) M-PEG-qdot (34 nM) plus 34 nM PEG-qdot	140.10 ± 86.82	18.02 ± 21.11
14	Fig. 9B	ρ1 ^C	(1) GABA (100 μM), (2) M-PEG-qdot plus 100 μM GABA	22.41 ± 32.71	7.32 ± 12.30
15	Fig. 9C	Same ρ (14) ^C	(1) GABA (100 μM), (2) M-PEG-qdot plus 100 μM GABA, (3) wash, and M-PEG-qdot (34 nM)	119.51 ± 84.47	11.83 ± 15.01

^AData indicated in the Border and Background columns of each row are the means ± SDs of fluorescence intensities determined with a 0–255 gray scale.

^BResults shown in rows 4–6 are aggregate data determined in the indicated number of experiments. For all other rows, the data indicate results obtained in a single experiment; each of these experiments is illustrated as denoted in the “Figure” column.

^CData indicated in rows 11, 13 and 15 were obtained from the same oocytes as those described in rows 10, 12 and 14, respectively.