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Article

Specific Fluorescent Probe Based on "Protect−Deprotect" To Visualize the Norepinephrine Signaling Pathway and Drug Intervention Tracers

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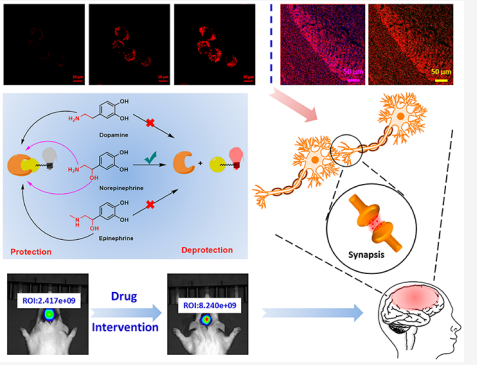
ABSTRACT: In recent years, increased social pressure and other factors have led to a surge in the number of people suffering from depression: studies show that quite a few people will experience major depression in their lifetime. Currently, it is widely believed that the internal cause of major depression is reduced levels of norepinephrine (NE) in brain tissue. Norepinephrine is very similar in structure and chemical properties to the other two catecholamine neurotransmitters, epinephrine (EP) and dopamine (DA). These three neurotransmitters are synthesized sequentially through enzymatic reactions in the biological system. Therefore, design of a norepinephrine-specific fluorescent probe is very challenging. In this work, we utilized a "protect−deprotect" strategy: longer emission wavelength cyanine containing water-soluble sulfonate was protected by a carbonic ester linking departing group thiophenol; the β-hydroxy ethyl amine moiety of norepinephrine may react with the carbonic ester via nucleophilic substitution and intramolecular nucleophilic cyclization to release the fluorophore. The process realized the specific red fluorescence detection of norepinephrine. Imaging of the norepinephrine nerve signal transduction stimulated by potassium ion was studied. More importantly, real-time fluorescence imaging of norepinephrine levels in the brain of rats stimulated by antidepressant drugs was studied for the first time.



INTRODUCTION

The signaling mode between neurons is mainly dependent on the secretion, transmission, and uptake of neurotransmitters. Neurotransmitters are chemicals released by neurons that bind to specific receptors in specific ways. Interactions between neurotransmitters and receptors are usually transient, lasting from milliseconds to minutes. However, its effects can make long-term changes in the target cell lasting for hours or days, which can have a huge impact on the body.[1] In recent years, with the increase of social pressure, the number of depressive symptomatology patients has soared. According to the World Health Organization, the number of people suffering from depression globally has exceeded 264 million. Depression has become the leading cause of disability worldwide and a major contributor to the global burden of disease.[2] Studies have shown that depression is closely related to decreased levels of norepinephrine (NE) in the brain.[3] Norepinephrine, as the central substance in the synthesis of monoamine catecholamine neurotransmitters, is produced by the enzymatic reaction of dopamine (DA) and then epinephrine (EP) under the action of phenylethanolamine *N*-methyltransferase (PMNT).[4,5] It can be seen that the structures and properties of the three monoamine neurotransmitters are similar and synthesized successively through enzymatic reactions. It is an extremely difficult task to design fluorescent probes that can respond specifically to norepinephrine.

Traditional methods for the detection of neurotransmitters mainly rely on electrochemical analysis and mass spectrometry, However, they often have some limitations in situ in vivo.[6−9]

At present, the fluorescence method for the detection and marking of norepinephrine mainly included the following aspects. The Kleinfeld team reported the cell-based neurotransmitter fluorescently engineered reporters; CNiFERs was used for fluorescence imaging of changes in NE or DA extracellular concentration during nerve signal transduction. The system indirectly detected the release of neurotransmitters using Ca^{2+} fluorescent probes to detect the concentration of intracellular Ca^{2+} during exocytosis.[10] Sames reported imaging the release of NE during nerve signal transduction using a pseudoneurotransmitter fluorescent dye FFN270 specifically labeled with norepinephrine cells.[11] Li reported that the specific binding of NE and GPCR was used to regulate the

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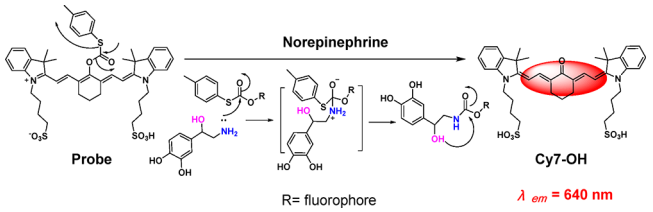
deprotonation process of cpEGFP connected with GPCR, and then an enhanced single-channel fluorescence signal was released to realize specific, highly sensitive, and real-time monitoring of NE.[12] The Glass group research has long been concentrated on using small organic molecules for the fluorescence detection of neurotransmitters using the aldehyde group conjugated with the fluorophore as the reaction site, which has different bonding constants with the primary amine of targets resulting in different fluorescence signal changes.[13,14]

Therefore, detection of the neurotransmitter content and release markers by fluorescent probes based on a norepinephrine-specific response is still a challenge. In our previous study, we initially found that the unique 2-aminoethanol moiety of norepinephrine had a cascade nucleophilic substitution with carbonates to release the fluorophore, thus realizing the specific detection of norepinephrine. Moreover, it was successfully applied to the specific labeling of norepinephrine in brain tissue slices through dual immunofluorescent probes.[15] However, the water solubility and short emission wavelength of the probe limited its further application.

In this work, we realized red fluorescence detection for norepinephrine in an aqueous solution using cyanine as a fluorophore to lengthen its emission wavelength and introduce sulfonate to greatly improve its water solubility (Scheme 1).

Scheme 1. Response Mechanism of the Probe with Norepinephrine



Subsequently, the probe successfully labeled norepinephrine-rich vesicles in PC12 cells and imaged the process of norepinephrine exocytosis when stimulated by high potassium. For the first time, in situ imaging of norepinephrine elevation in vivo induced by fluoxetine (a typical antidepressant) has been achieved. It will have certain practical significance for the research and screening of depression drug therapy.

RESULTS AND DISCUSSION

Synthesis and Characterization. Compounds were synthesized according to the procedure in Scheme S1 in the Supporting Information. Eventually the probe was obtained as a dark green solid (0.18 g, 21%). ^{1}H NMR (600 MHz, CD_3OD) δ 7.85 (d, J = 14.0 Hz, 2H), 7.52 (d, J = 7.3 Hz, 2H), 7.48 (d, J = 7.7 Hz, 2H), 7.39 (t, J = 7.6 Hz, 2H), 7.34 (d, J = 7.9 Hz, 2H), 7.30 (d, J = 7.6 Hz, 2H), 7.25 (t, J = 7.3 Hz, 2H), 6.25 (d, J = 14.1 Hz, 2H), 4.17 (t, J = 6.7 Hz, 4H), 2.85 (t, J = 6.7 Hz, 4H), 2.68 (s, 4H), 2.35 (s, 3H), 1.99−1.88 (m, 10H), 1.73 (s, 12H); ^{13}C NMR (151 MHz, CD_3OD) δ 144.1, 104.5, 54.3, 51.9, 51.8, 51.6, 51.5, 51.3, 51.2, 51.1, 47.5, 31.1, 29.8, 24.6 (Figure S1). HR-MS: calcd 857.29696; found 857.29698 (Figure S2).

Norepinephrine Detection Using a Probe. The specific response mechanism of the probe to norepinephrine was confirmed again by mass spectrometry. In the final reaction products both the cyanine ketone peak at 353.13792 $[Cy7\text{-}OH]^{2-}$ and the five-membered ring compound peak 194.04537 $[M - H]^-$ were found in the HR-MS (Figure S3). Subsequently, the UV−vis and fluorescence response of the probe to norepinephrine in vitro was then carried out. Since the water solubility of the probe was greatly improved, we carried out spectrometric determination in the PB system (pH 5.0). Five millimolar NE was added to 2 mL of PB (pH 5.0) containing a 10 μM probe. As shown in Figure 1a, the UV

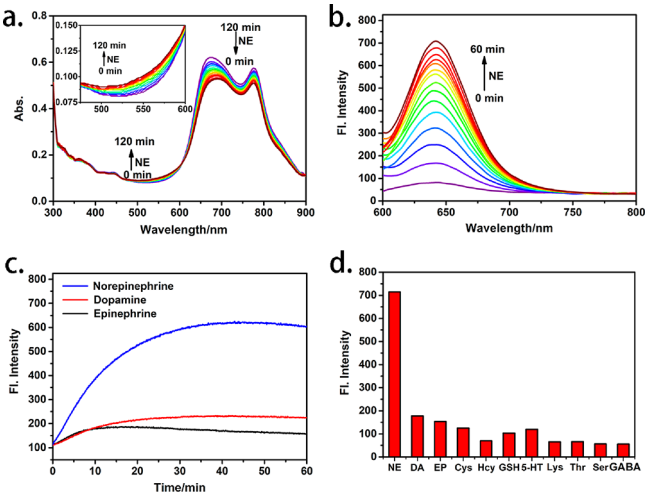


Figure 1. (a) UV−vis response of a 10 μM probe upon 5 mM NE in PB (pH 5.0) for 0−120 min. (b) Fluorescent response of a 10 μM probe upon 5 mM NE in PB (pH 5.0) for 0−60 min (λ_{ex} = 550 nm, slit = 10 nm/5 nm). (c) Time dependence on a 10 μM probe upon 5 mM NE, DA, and EP for 0−60 min. (d) Selectivity of a 10 μM probe upon 5 mM neurotransmitters (DA, EP, NE, 5-HT, GABA) and GSH, 500 μM amino acids (lys, thr, ser), 100 μM Cys, and 10 μM Hcy.

absorption of the system at 675 and 775 nm gradually decreased over time, while that at 550 nm gradually increased. Accordingly, after addition of NE, the fluorescence intensity of the system gradually increased at 640 nm, which was 14 times higher than that of the system containing only the probe (Figure 1b). The spectral properties of the system after reaction were in good agreement with those of the cyanine ketone itself (Figure S4).

In further experiments, as we designed, both dopamine and epinephrine still induced less fluorescent intensities enhancement compared with norepinephrine (Figures 1c). Besides we also studied other neurotransmitters (5 mM), such as 5-HT, GABA, and amino acids (500 μM), such as threonine, serine, and lysine. Studies have shown that none of them induced a distinct fluorescent change of the probe in the detection system (Figures 1d and S5). We also investigated 100 μM cysteine/10 μM homocysteine with a similar mercaptoethylamine moiety and 5 mM GSH, which all did not induce a significant fluorescent response (the cellular concentration of unbounded Cys/Hcy is nnanomolar to micromolar, Figure 1d). The probe response time in this work was greatly shortened, and the reaction was completed within 50 min so as to minimize the loss of norepinephrine caused by REDOX.

Imaging of Endogenous Norepinephrine in Living Cells. As a neurotransmitter, norepinephrine is usually concentrated in specialized cells or tissues. We selected several kinds of cells to mark endogenous norepinephrine. When the 20 μM probe was cultured with Hela cells and HepG-2 cells for 40 min at 37 °C, no fluorescence emission was observed in the

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red channel (Figure 2). PC12 cells are often used to build neural models because of its characteristics of nerve cells and

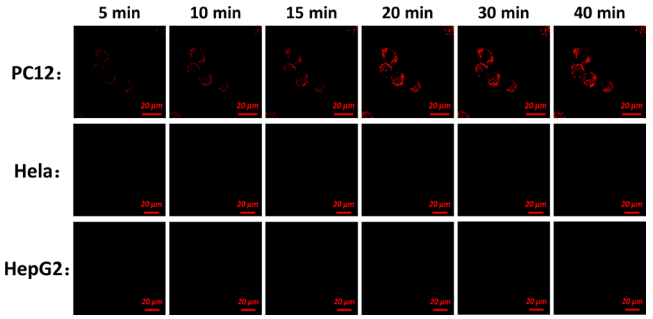

Figure 2. PC12 cells, Hela cells, and HepG2 cells with the probe (20 μM) cultured in the red channel (5−40 min) (λ_{ex}: = 561 nm, bar = 20 μm).

secretory cells. Studies have shown that PC12 cells can secrete norepinephrine-rich vesicles and have exocytosis.[16] Surely, after the probe was cultured with PC12 cells in similar conditions, an obvious red fluorescence emission could be observed and it increased over time (Figure 2). The results showed that the probe could mark the norepinephrine in the vesicle.[17]

Imaging of the Process of Norepinephrine Exocytosis. The study of signal transduction of neurotransmitters is beneficial to the prevention and intervention of neurological diseases. It is reported that the action potential produced by high concentrations of potassium stimulating the cell causes voltage-dependent calcium-ion channel depolarization to open the calcium-ion channels. Driven by the electrochemical potential, calcium ions flow into cells from the outside; the increased concentration of calcium in the cytoplasm triggers fusion of vesicles with the cytoplasmic membrane. Consequently, it stimulates the release of neurotransmitters.[18] In this work, PC12 cells loaded with probes-labeled norepinephrine vesicles were stimulated with high-K^+ solution, and the intensity of the fluorescent spots in the cells gradually decreased. However, under the same conditions, the cells were stimulated with PBS (without potassium ion). The fluorescent intensity was almost unchanged in the red channel (as shown in Figure 3). The results indicated that increased K^+ concentration led to cell exocytosis and that the probe realized visualization of the norepinephrine signaling pathway.

Noradrenaline Visualization in Brain Tissue. As a long-wavelength red emission fluorescent probe, we used it for brain tissue labeling. Noradrenergic neurons are distributed in the brainstem nucleus locus coeruleus. Dual immunolabeling for TH and DBH or DBH and PNMT is a successful and effective method. In this work, we still employed a primary antibody cocktail of Rabbit Anti-Tyrosine Hydroxylase Polyclonal antibody (1:1000, bs-0016R; Bioss Antibodies) and Anti-Dopamine β-Hydroxylase (1:1000, MAB308; Millipore) for TH and DBH dual-labeling experiments for 2 h. This was followed by a secondary antibody cocktail of Goat Anti-Rabbit IgG/Cy3 (1:250, bs-0295G-Cy3; Bioss Antibodies) and Goat Anti-Mouse IgG/ALexa Fluor 350 (1:100, bs-0296G-AF350; Bioss Antibodies) for 30 min, which were then stained with the probe (10 μM) for 120 min. For PNMT and DBH dual-labeling experiments, the sections were processed with a primary antibody cocktail of Rabbit Anti-PNMT Polyclonal antibody (1:1000, bs-3912R; Bioss Antibodies) and Anti-

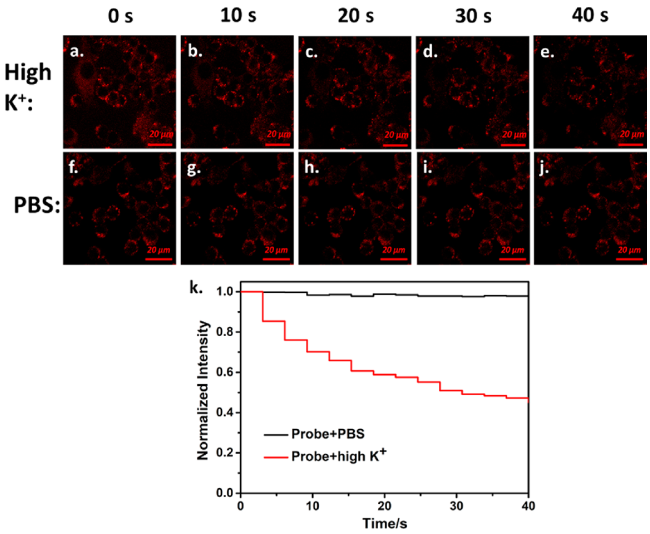

Figure 3. (a−e) Probe-loaded PC12 cells with high K^+ (100 μL) in the red channel (0−40 s). (f−j) Probe-loaded PC12 cells with PBS in the red channel (0−40 s). (k) Time dependence on probe-loaded PC12 cells with high K^+ (100 μL) and PBS in 0−40 s (λ_{ex} = 561 nm, bar = 20 μm).

Dopamine β-Hydroxylase (1:1000, MAB308; Millipore) for 2 h followed by a secondary antibody cocktail of Goat Anti-Rabbit IgG/Cy3 (1:150, bs-0295G-Cy3; Bioss Antibodies) and Goat Anti-Mouse IgG/ALexa Fluor 350 (1:100, bs-0296G-AF350; BiossAntibodies) for 30 min, which were then stained with the probe (10 μM) for 120 min. As shown in Figure 4, the

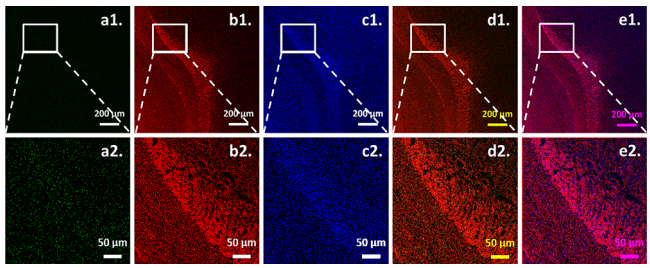

Figure 4. (a1, a2) Immunofluorescence the Cy3-labeled TH-positive region. (b1, b2) Probe-labeled brain tissue. (c1, c2) Immunofluorescence AF350-labeled DBH-positive region. (d1) Merged image of a1 and b1. (d2) Merged image of a2 and b2. (e1) Merged image of b1 and c1. (e2) Merged image of b2 and c2 (Cy3: λ_{ex} = 548 nm. AF350: λ_{ex} = 405 nm. Probe: λ_{ex} = 561 nm. Bar = 200 μm).

area involving the probe (b1, b2) and DBH-AF350 (c1,c2) fluorescent emission showed extensive overlap (e1, e2), which indicated that the probe has a specific labeling ability for noradrenergic neurons. Also, it still showed the probe specifically labeled norepinephrine over epinephrine by dual immunolabeling for DBH and PNMT (Figure 5). This bidirectional detection strategy strongly proved that the probe can efficiently and specifically label norepinephrine in the tissue level.

Fluorescent Imaging In Vivo. The penetration depth of imaging is always limited by the wavelength due to the long wavelength of the probe effectively supplementing some of the limitations of fluorescent imaging applications; we carried out fluorescent in vivo imaging. The probe (20 μM) was injected on the right side of the back of mice, and then norepinephrine

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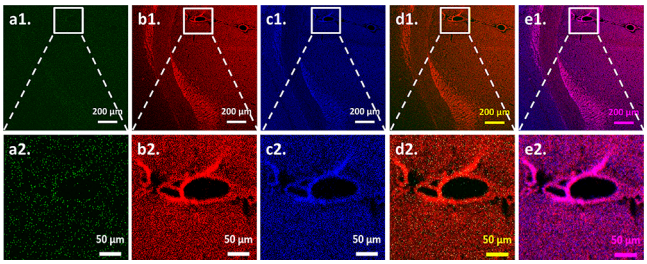

Figure 5. (a1, a2) Immunofluorescence the Cy3-labeled PNMT-positive region. (b1, b2) Probe-labeled brain tissue. (c1, c2) Immunofluorescence AF350-labeled DBH-positive region. (d1) Merged image of a1 and b1. (d2) Merged image of a2 and b2. (e1) Merged image of b1 and c1. (e2) Merged image of b2 and c2 (Cy3: λ_{ex} = 548 nm. AF350: λ_{ex} = 405 nm. Probe: λ_{ex} = 561 nm. Bar = 200 μm).

(200 μM) was injected in the same area. The probe (20 μM) and PBS (same volume with NE) were injected on the other side of the back of mice. As shown in Figure 6, the signal of the

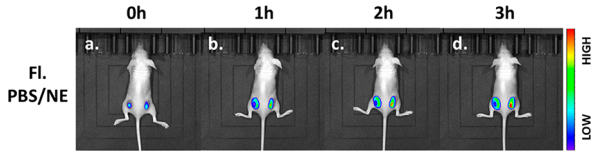

Figure 6. In vivo fluorescent imaging of mice injected with PBS/NE (λ_{ex} = 561 nm).

NE group increased in the red channel over time, but there was no significant change in the control group. We believe that the probe can be considered as an effective tool for detecting NE in vivo as a good clinical application prospect in quantitative analysis of neurotransmitters and signal pathways.

Norepinephrine Release Imaging with Fluoxetine. Studies have shown fluoxetine as a selective serotonin reuptake inhibitor can enhance serotonin neurotransmission to produce antidepressant effects as fluoxetine,[19] and it can also increase dopamine and extracellular norepinephrine levels in the hypothalamus,[20] cortex,[21] and prefrontal cortex.[22] Currently, fluoxetine is an effective clinical antidepressant on basal and stress-induced norepinephrine (NE) release.[23] Therefore, in the following experiment, we carried out the norepinephrine release imaging experiment with fluoxetine intervention. We injected fluoxetine (10 mg/kg) intraperitoneally into the rats; after 40 min of surgical exposure of the brain,[24] the probe (200 μM) was directly added to the brain and then imaged. Compared with the rats given the same volume of saline intraperitoneally, the rats treated with fluoxetine had significantly higher norepinephrine levels in the brain as the control group (Figure 7). The results provided scientific data for the screening and efficacy of antidepressant drugs in a clinic in the future. At this point, we will study the intervention and treatment of various antidepressants under all kinds of depression through specific fluorescent probe bioimaging and do in-depth research on the mechanism so as to provide a powerful database for the development of effective antidepressants.

CONCLUSION

In view of the challenge of developing specific NE probes, we used this strategy: the fluorophore protected by the leaving

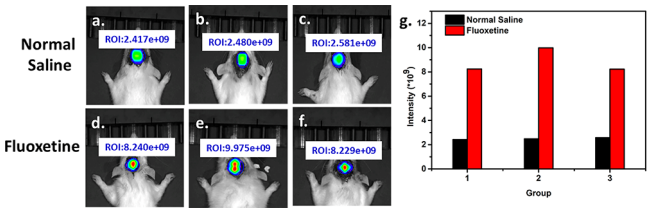

Figure 7. (a–c) In vivo imaging of rats intraperitoneally injected with saline. (d–f) In vivo imaging of rats intraperitoneally injected with fluoxetine (10 mg/kg). (g) Fluorescent intensity of ROIs normal saline and fluoxetine injected rats (λ_{ex} = 561 nm).

group produces the intramolecular PET effect resulting in no fluorescence emission. Subsequently, using the unique amino ethanol structural unit of NE, through cascade nucleophilic and leaving reactions, deprotection and release of the fluorophore occurs. Using the fluorescent probe with a long-wavelength red emission, we verified the specific response of the probe to NE in a complex biological environment through cell imaging, slice imaging, and in vivo imaging. In this work, we applied the probe for fluorescence imaging of norepinephrine exocytosis signaling pathways under high K^+ stimulation. The first visualization of the effect of antidepressant drugs on norepinephrine intervention was achieved.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c08956.

Experimental details and additional spectroscopic data (PDF)

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Author Contributions

This manuscript was written through contributions from each author. Each author approved the final version of this manuscript.

Notes

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