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Toward live-cell imaging of dopamine neurotransmission with fluorescent neurotransmitter analogues†

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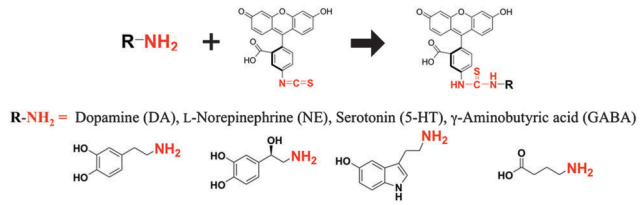
We report a novel '*fluorescent dopamine*' that possesses essential features of natural dopamine. Our method is simple and is readily extended to monoamine neurotransmitters such as L-norepinephrine, serotonin and GABA, providing a more practical approach. Because of its compatibility with sensitive fluorescent measurements, we envisage that our approach will have a broad range of applications in neural research.

Neurotransmitters are chemicals that serve as messengers in neurotransmission.[1] They are packaged into synaptic vesicles of a neural cell by transporters on the vesicular membrane; when an action potential arrives, they are released from synaptic vesicles to the extracellular space to trigger another neural cell or an effector cell upon binding to the receptors of the target cell. The interaction between neurotransmitters and receptors is terminated mainly through reuptake of neurotransmitters back into the cytosol of a neural cell by transporters on the plasma membrane. Disorders of the production, release, reception, breakdown or reuptake of neurotransmitters can cause various neurologic or psychiatric diseases.[2] In particular, a loss of dopaminergic neurons is associated with Parkinson's disease.[3]

Progress in understanding the molecular basis of neurotransmission will benefit from approaches that allow one to probe the release and reuptake of specific neurotransmitters at the level of single living cell with great sensitivity and selectivity. Among many approaches that enable the determination of neurotransmitters,[4] fluorescent imaging is of particular interest because of its great sensitivity and spatiotemporal resolution and its compatibility with contemporary protocols for both living cells and animals. The design of various fluorescent probes to mimic neurotransmitters has accordingly emerged as a strategy for the investigation of neurotransmission.[5–7]



Scheme 1 Construction of fluorescent analogues of neurotransmitters.

We employ a simple strategy to construct fluorescent analogues of neurotransmitters through direct conjugation of a dye molecule to the neurotransmitter (Scheme 1). We synthesized fluorescent analogues of dopamine (DA), L-norepinephrine (L-NE), serotonin (5-HT) and γ-aminobutyric acid (GABA). The details of synthesis, characterization and experimental methods are described in the ESI.†

We particularly characterized the fluorescent analogue of dopamine relative to natural dopamine using rat adrenal pheochromocytoma cells (the PC12 cell), a model cell line commonly utilized to study the synthesis and release of dopamine neurotransmitters.[8] We showed that the '*fluorescent dopamine*' exhibits numerous essential features of its natural counterpart: it is accumulated selectively by PC12 cells, but not by other cells that lack monoamine transporters. This distinct selectivity of reuptake is mediated mainly by monoamine transporters. It acts as a substrate of the vesicular monoamine transporter; its release can be evoked by potassium ions through a pathway dependent on calcium ions. Moreover, it enables a selective mapping of excised brain tissues. Although this work focuses predominantly on dopamine, the same strategy can potentially be generalized to other neurotransmitters that contain a primary amine such as L-NE, 5-HT and GABA. As our synthesis requires no laborious process, it provides a practical approach for those who seek to employ such a tool to study neuronal cell function with limited equipment and expertise in synthesis.

We first tested the uptake of various fluorescent analogues by PC12 cells. Among these fluorescent analogues, FITC-DA, FITC-L-NE and FITC-5-HT accumulated excessively in PC12 cells; in contrast, the uptake of FITC-GABA was not discernible relative to an untreated control (Fig. 1a and b). Besides, PC12

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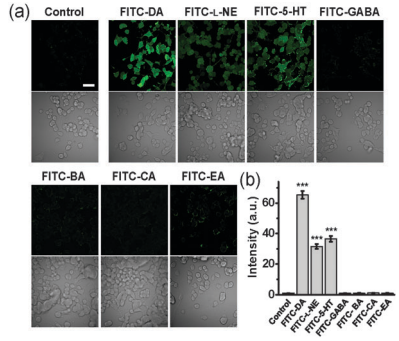

Fig. 1 Uptake of various fluorescent analogues by PC12 cells. (a) Fluorescence (upper row) and bright-field (lower row) images showing the uptake of fluorescent analogues of neurotransmitters and non-neurotransmitter monoamines (control: cells treated with a medium having no fluorescent analogue). Scale bar: 40 μm. (b) Quantitative analysis of the images demonstrating the selective uptake of FITC-DA, FITC-L-NE and FITC-5-HT by PC12 cells. ***$P < 0.001$ (n = 30).

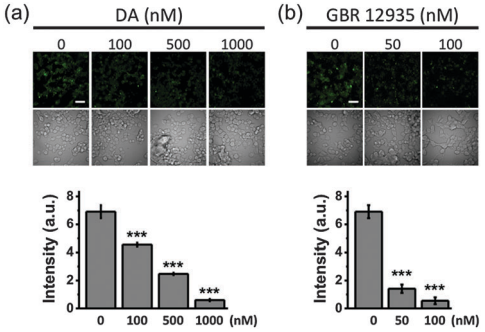

Fig. 2 Mechanistic study of the internalization of FITC-DA into PC12 cells. (a) Microscopic images and quantitative analysis showing the suppressed uptake of FITC-DA with cotreatment of natural dopamine. (b) Results showing the suppressed uptake of FITC-DA with pretreatment of GBR 12935. ***$P < 0.001$ (n = 30). Scale bar: 40 μm.

cells treated with FITC-DA exhibited numerous punctate structures, which conform to the feature of neurotransmitter vesicles (Fig. S1, ESI†). DA, L-NE and 5-HT are generally classified in the monoamine family of neurotransmitters,[9] whereas GABA belongs to the amino-acid family of neurotransmitters.[10] Our result thus indicates that the fluorescent analogues of monoamine neurotransmitters were preferably accumulated in PC12 cells; this result is consistent with the expression of monoamine transporters in PC12 cells.[11]

We investigated particularly the structural aspects of the selective uptake of the '*fluorescent dopamine*'. Some, but not all, FITC-conjugates accumulated significantly in PC12 cells. The selective uptake is hence unlikely to be facilitated predominantly by the FITC moiety. To test whether the dopamine moiety is essential in the selective uptake, we synthesized fluorescent conjugates of various monoamines: benzylamine (BA), cyclohexylamine (CA) and ethylamine (EA). These monoamines are structurally similar to dopamine, but are not neurotransmitters. We then examined their uptake by PC12 cells; these fluorescent conjugates did not accumulate in PC12 cells (Fig. 1a and b). The result indicates that the dopamine moiety of FITC-DA is essential in the selective uptake of the fluorescent analogues of dopamine by PC12 cells.

To acquire further mechanistic insight into this selective uptake, we examined how FITC-DA entered the plasma membrane of PC12 cells. A cotreatment of dopamine at an equal amount (100 nM) resulted in a significantly decreased uptake of FITC-DA (34% decrease); the suppression was enhanced with an administration of more dopamine (64% and 91% decrease relative to the untreated control for treatments at 500 nM and 1000 nM dopamine, respectively) (Fig. 2a). We then examined the effect of GBR 12935, an effective blocker of monoamine transporters,[12] on the internalization of FITC-DA by PC12 cells. We loaded in sequence GBR 12935 (50 nM, 90 min) and then FITC-DA (100 nM, 10 min) onto PC12 cells. The uptake of FITC-DA decreased significantly (80% decrease relative to the control), and for a greater dose a greater suppression (92% decrease for a treatment of 100 nM) (Fig. 2b). These collective results indicate that FITC-DA was internalized into PC12 cells predominantly through the monoamine transporter on the plasma membrane of cells. More importantly, they indicate that the structural characteristic essential for the recognition and internalization by corresponding transporters is largely maintained in FITC-DA despite the conjugation. These deductions also explain the selective uptake of other '*fluorescent monoamine neurotransmitters*' (FITC-L-NE and FITC-5-HT) by PC12 cells as shown in Fig. 1.

Our preceding results show that the fluorescent analogue of dopamine possesses some characteristics of natural dopamine with respect to its uptake by PC12 cells. This finding and the possibility to detect or to image FITC-DA in cells or tissues can stimulate new possibilities in neuronal research. We demonstrate them in subsequent paragraphs.

We first tested the cytotoxicity of FITC-DA by examining whether the treatment of FITC-DA impairs the differentiation of PC12 cells. We preloaded PC12 cells with FITC-DA and incubated them with neural growth factor (NGF) for a duration of 96 h. The control corresponded to PC12 cells incubated with NGF for the same duration, but not preloaded with FITC-DA. The result shows that the cells pretreated with FITC-DA continued to grow normally with numerous dendrites and axons protruding from the soma of the cells in a way similar to that of the control (Fig. S2, ESI†). This observation demonstrates that FITC-DA did not impair the differentiation of PC12 cells and imposed negligible cytotoxicity.

Having shown that FITC-DA accumulated abundantly in monoaminergic PC 12 cells, we proceeded to demonstrate the application of FITC-DA for selective labeling of cells with catecholamine transporters. We tested cells of several types. Among them, HepG2 and HeLa are not neural cell lines, and NB41A3 is a mouse neuroblastoma cell line that possesses some characteristics of mature neurons.[13] To our knowledge, none of these cell lines expresses catecholamine or monoamine transporters. We also tested SK-N-SH, a human catecholaminergic neuroblastoma cell line that expresses catecholamine transporters.[14] As shown in the result (Fig. 3), FITC-DA accumulated excessively in SK-N-SH cells (and in PC12 cells as shown before), but not appreciably in the cells of the other three types. This result shows convincingly that FITC-DA can serve as a selective fluorescent probe to label catecholaminergic and monoaminergic cells.

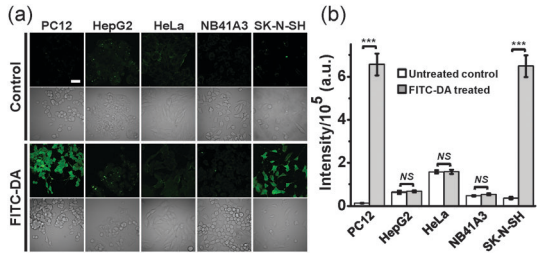

Fig. 3 Uptake of FITC-DA by cells of various types. (a) Fluorescent (green) and bright-field (grey) images of the untreated control and the cells loaded with FITC-DA. (b) Results of quantitative analysis. ***$P < 0.001$, *NS*: not significant ($n = 25$). Scale bar: 40 μm.

To demonstrate a quantitative assessment of the evoked release of dopamine, we added potassium ions to PC12 cells that were preloaded with FITC-DA. We recorded continuously the temporal fluorescent intensity with a confocal setup (Fig. 4a), which ensured that the recorded fluorescence was produced exclusively from the intracellular space. Our results show that the treatment of potassium ions caused a greatly decreased intensity of fluorescence (blue, Fig. 4b). This observation contrasts with that obtained from either the untreated control (light grey, Fig. 4b) or the sham experiment (dark grey, Fig. 4b), which exhibited negligibly altered fluorescent intensity. For comparison, we tested cells preloaded with reserpine, a blocker of vesicular monoamine transporter 2 (VMAT2),[15] or with cadmium ions, a blocker of the calcium channel.[16] Either pretreatment effectively inhibited a K^+-evoked secretion of FITC-DA (blue, Fig. 4c and d). These collective results show that FITC-DA behaves in a way similar to natural dopamine with respect to the stimulated secretion.

We proceeded to demonstrate a selective mapping of intact brain tissues of rats. As shown from the result (Fig. 5), the tissue incubated with FITC-DA exhibited a clear contrast. In particular, the fluorescence in the region corresponding to the corpus callosum (CC) was dim, whereas the regions corresponding to the cortex (CTX) and striatum (STR) exhibited bright fluorescence. The intensity in the striatum was slightly greater than that in the cortex. In contrast, the control shows no discernible fluorescence.

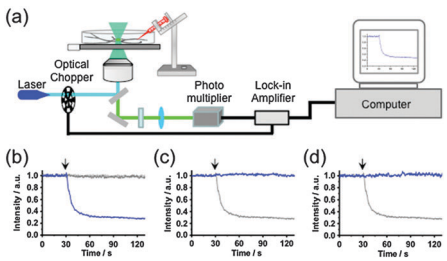

Fig. 4 Stimulated release of FITC-DA. (a) Schematic of the setup. (b) Temporally varying fluorescence showing a secretion of FITC-DA from single cell (blue: stimulation with K^+ ion; light grey: the untreated control; grey: sham experiment implemented with an injection of PBS). (c) The pretreatment of reserpine to cells precluded K^+-stimulated release of FITC-DA (blue), in contrast with the result on cells stimulated with K^+ alone (grey). (d) The cotreatment of Cd^{2+} inhibited K^+-stimulated release (blue) relative to the control (cells stimulated with K^+ alone, grey).

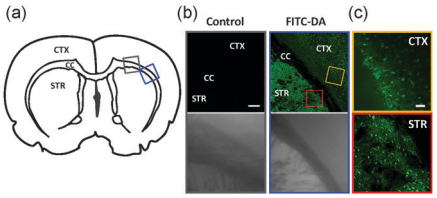

Fig. 5 Selective mapping of acute brain slices with FITC-DA. (a) Sketch illustrating the approximate regions of the brain slice at which the images were acquired. (b) Fluorescence (upper row) and bright-field (lower row) images showing that FITC-DA stained selective regions of the brain slice. The control was obtained from a brain slice incubated in a medium containing no FITC-DA. Scale bar: 200 μm. (c) Magnified images of the marked areas in (b). CTX: cortex; CC: corpus callosum; STR: striatum. Scale bar: 40 μm.

These findings conform to a preceding knowledge of the heterogeneous innervation of dopaminergic systems in the brain. In particular, the corpus callosum is known to lack monoamine terminals and neuronal cell bodies;[5] consistently, we observed little accumulation of FITC-DA in the region corresponding to the corpus callosum. Moreover, the appreciable accumulation of FITC-DA in the regions corresponding to the striatum and cortex conforms to the established dopaminergic innervation in these regions.[17]

Beyond acting as a neurotransmitter, dopamine is known to act as a hormone that exerts a positive inotropic effect and a chronotropic effect at small and large doses.[18] To examine whether FITC-DA possesses a pharmacological activity similar to that of natural dopamine, we measured the heart rate of rats that were injected intravenously either with DA or with FITC-DA of the same dose. An intravenous injection of FITC-DA caused a significantly elevated heart rate relative to the untreated control (310 ± 10.4 *vs.* 227 ± 7.1 beat per min; $n = 5$, $P < 0.001$); this increase agrees quantitatively with the result obtained with dopamine at the same dose (304 ± 7.6 beat per min; $n = 5$) (Fig. S3, ESI†). In contrast, an injection of normal saline (226 ± 9.4 beat per min; $n = 5$, $P = 0.924$), normal saline containing only FITC (229 ± 5.0 beat per min; $n = 5$, $P = 0.870$) or normal saline containing only FITC-EA (233 ± 5.0 beat per min; $n = 5$, $P = 0.510$) resulted in no significant change of the heart rate relative to the untreated control. These results show clearly that, similar to dopamine, FITC-DA exhibited a pharmacological activity *in vivo* acting as a positive chronotrope; furthermore, the result indicates that FITC-DA and DA trigger the same receptor to stimulate the heart rate.

We have devised a fluorescent analogue of dopamine to facilitate the detection and imaging of dopamine in cells and tissues. Sulzer and Sames developed numerous fluorescent false neurotransmitters and demonstrated the visualization of the release of neurotransmitters from individual synaptic terminals.[5,7] Unlike their approach that generally requires synthesis of numerous trial compounds followed by screening of their activities, our '*fluorescent dopamine*' is synthesized through a direct conjugation of a fluorescent dye to a natural dopamine. This condition is important because our method is simple and straightforward, providing a more practical approach (especially for those with limited equipment and expertise in synthesis who seek to employ such

a tool to study cell function). Specifically, in the work of Sulzer and Sames, the aminoethyl group of dopamine was conserved whereas the aromatic system was expanded to engineer the desired fluorescent properties in a laborious process. Here we simply coupled FITC to the primary amine of several neurotransmitters, and showed that FITC-DA acts as a substrate of monoamine transporter.

We note that it has been possible to image directly the serotonin and dopamine neurotransmitters in live neurons without labeling on using multiphoton microscopy,[19] but a necessity to use costly pulsed lasers might hinder the adaption of their approach by other research groups. Besides, phototoxicity or photochemical damage might be a concern as a large power of excitation (100 times that commonly employed for single-photon imaging) is generally employed for multiphoton imaging.

This work focuses on dopamine. To test whether the same approach is readily extensible to other monoamine neurotransmitters such as L-norepinephrine, serotonin and GABA, we examined their fluorescent analogues on three different cell lines (PC12, CHO-K1 and SK-N-SH) (Fig. S4, ESI†). Firstly, conforming to the expression of L-NE transporters in PC12 and SK-N-SH cells,[20] and the deficiency of L-NE transporters in CHO-K1 cells, FITC-L-NE accumulated in both PC12 and SK-N-SH cells, but not in CHO-K1 cells. Secondly, FITC-5-HT accumulated abundantly in PC12 cells relative to CHO-K1 and SK-N-SH cells, consistent with the expression of 5-HT transporters on PC12 cells[21] and the deficiency of 5-HT transporters in SK-N-SH and CHO-K1 cells.[22] Finally, the accumulation of FITC-GABA in these three types of cells is negligible as no such cell line is known to express GABA transporters. Taken together, these results indicate that our approach is extensible to other monoamine neurotransmitters.

Our results show that a molecule as large as FITC-DA can traverse monoamine transporters on plasma membrane and vesicular monoamine transporters. This result indicates that our understanding of transporter mechanisms might be incomplete. Particularly, important questions such as which dominant moiety of dopamine governs its recognition and the internalization by transporters remain unanswered. For instance, whether the hydroxyl groups of the catechol moiety are essential in the uptake of dopamine is equivocal.[23] As *N*-alkylation of the amine moiety substantially decreased the uptake activity,[23] a primary amine might be favourable and the uptake activity might not be tolerant of substitutions of the amino group. However, MDMA (3,4-methylenedioxy-*N*-methylamphetamine), a compound structurally related to dopamine with a methyl substitution at the amine moiety, was shown to act as a substrate of the dopamine transporter.[24] Similarly, METH (*N*-methyl-1-phenylpropan-2-amine) acts as a substrate of the dopamine transporter, and is readily internalized into the nerve terminal.[25] Furthermore, dopexamine acted as a ligand of the dopamine receptor despite the possession of a bulky substitution at the amine group.[26] All these results strongly indicate a complicated interaction between neurotransmitters and transporters (or receptors). More sophisticated mechanistic studies are hence necessary in order to elucidate their structure–activity relation.

In summary, we have developed a novel fluorescent analogue of dopamine that enables fluorescence imaging and detection of neurotransmitters, and possesses multiple essential features of natural dopamine as a neurotransmitter. We envisage that our approach, with its compatibility with sensitive fluorescent detection and imaging, will have broad applications in research on chemical neurotransmission in living cells and tissues.

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