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Electroactive fluorescent false neurotransmitter FFN102 partially replaces dopamine in PC12 cell vesicles

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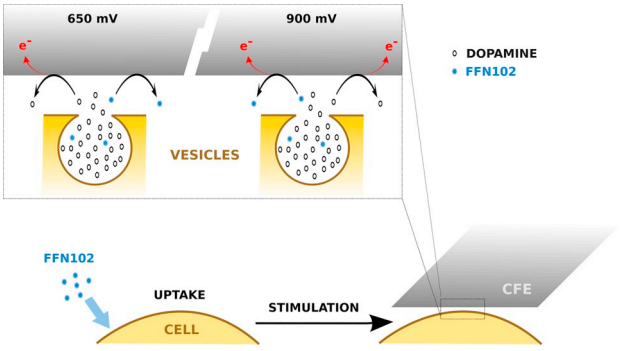
HIGHLIGHTS

- The uptake of the Fluorescent False Neurotransmitter FFN102 in PC12 cells vesicles was investigated using amperometry.
- The FFN102 replaced ca. 10% of the dopamine stored in the PC12 cells vesicles.
- The FFN102 enriched the fast transport compartment of the PC12 cells vesicles.
- The upload of FFN102 did not modify the secretion features of PC12 cells.
- The FFN102 was shown to be a suitable probe for bioanalytical and pharmacological studies at the secretory PC12 cells.

GRAPHICAL ABSTRACT



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ABSTRACT

In the last decade, following fluorescent dyes and protein tags, pH sensitive false fluorescent neurotransmitters (FFN) were introduced and were valuable for labeling secretory vesicles and monitoring exocytosis at living cells. In particular, the synthetic analog of neurotransmitters FFN102 was shown to be an electroactive probe. Here, we show that FFN102 is suitable to be used as a bioanalytic probe at the widely used PC12 cell model. FFN102 was uptaken in the secretory vesicles of PC12 cells, partially replacing the endogenous dopamine stored in these vesicles. The different oxidation potentials of dopamine and FFN102 allowed to determine that ca. 12% of dopamine was replaced by FFN102. Moreover, the FFN102 was found to be over released through the initial fusion pore suggesting that it was mostly uptaken in fast diffusion compartment of the vesicles.

1. Introduction

Exocytosis is a key mechanism of the communication between cells and appears as a central feature of neurotransmission and secretion. Membrane bound vesicles containing chemical messengers fuse with the plasma membrane allowing the release in the extra-cellular medium. This release by the mean of quantal events [1] was experimentally suggested by electron microscopy micrographs [2]. Even though electron microscopy could capture vesicle exocytosis [3], dynamic imaging of exocytosis required the vesicles labeling and a higher time resolution that could be achieved by fluorescence microscopy. Accordingly two main classes of fluorescent imaging probes were

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introduced: fluorescent dyes and protein pH-sensitive tags. The former includes acridine orange used to stain the vesicle lumen [4]; the neutral acridine orange molecules cross the lipid membranes and concentrate in the acidic lumen of the vesicles as cations exhibiting enhanced fluorescence [5]. The membrane staining of the vesicles was achieved using styryl dyes, with the emblematic FM family [6]; these amphiphilic dyes partition between the medium and the cellular membrane. The fluorescence is significantly increased through solvatochromism for membrane embedded dyes. The styryl dyes uptake is then performed through endocytosis. A major shortcoming of the previous fluorescent dyes lies in the lack of specificity in membranes staining leading to a strong background. The signal to noise ratio of vesicles staining is improved with genetically encoded fluorescently labeled vesicular proteins [7]. The genetically encoded pHluorins, a pH-sensitive variant of the green fluorescent protein, was shown to report fusion pore opening and secretion of secretory granules when linked to vesicular proteins. Indeed, the fluorescence of these probes is quenched in the acidic lumen of the vesicles [8]. As an alternative approach, fluorescent false neurotransmitters (FFN) have been introduced to target vesicles through vesicular monoamine transporters (VMAT) [9]. Thereafter, pH-sensitive FFN have been designed [10] to report neurotransmitter release.

Parallel to the developments of these fluorescent imaging probes, electrochemical methods at single cell were introduced. These techniques have been shown effective at analyzing the nature of the vesicle release content through voltammetry at microelectrode [11]. Amperometry at microelectrode achieved to monitor exocytosis at single cell and to analyze individual exocytic events (kinetics and amount of released neurotransmitters) [12,13]. More recently, fusion pore expansion extent and rate were measured from single amperometric spikes [14]. The electroactivity of the pH-sensitive FFN102, a synthesized analogue of endogenous neurotransmitters, has been demonstrated and used in coupled monitoring of the secretion from BON N13 cells [15]. Previously, FFN102 had been shown valuable in sensing vesicular pH in PC12 cells [10]. The same molecule was then successfully used to identify and report activity of dopamine cells in brain tissue [16]. Contrary to pH-sensitive probes linked with vesicular proteins or peptides, small FFN like FFN102 exhibit rather low steric hindrance as the endogenous monoamine neurotransmitters. We report here on the loading of FFN102 in PC12 cells, a widely used model in fundamental investigation of exocytosis as well as for pharmacological and neurotoxical applications [17]. Compared to chromaffin cells obtained from animal, cultured PC12 cells have been recognized as an easy-to-use secretory cell model. We show that the uptake of FFN102 in PC12 secretory vesicles led to the replacement of a fraction of the endogenous dopamine stored in secretory vesicles without any other modification of the secretion process.

2. Material and methods

2.1. Reagents and solutions

FFN102 was synthesized in the lab according to the procedure reported by Lee et al. [10]. All aqueous solutions were prepared using 18.2 MΩ ultra-pure water (Milli-Q, Millipore). Unless stated, all chemicals were obtained from Sigma-Aldrich and used without further purification.

2.2. Cell culture and sample preparation

PC12 cells were purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in RPMI-1640 media, supplemented with 10% heated inactivated horse serum (Life technologies), 5% heated inactivated fetal bovine serum (Life technologies) and 1% penicillin streptomycin solution (Life technologies) in a 5% CO_2, 100% humidity atmosphere at 37°C. Cells were grown on cell culture flask with filter cap and were sub-cultured approximately every 4–5 days or when confluence was reached to almost 100%. Throughout the cell cultures lifetime, the medium was refreshed every 2 days.

Prior to electrochemical experiments, PC12 cells were grown on human placenta collagen (Bornstein and Traub type IV) coated 50 mm glass bottom dish (MatTek Corporation, Ashland, MA) 48 h in complete growth medium. Cells were cultured in a 20 μM FFN102 supplemented medium for 1 h before the experiment in order to achieve the active loading of secretory vesicles.

2.3. Fabrication of carbon fiber microelectrode (CFE)

The carbon fiber electrodes were fabricated by aspirating 10 μm diameter carbon fibers (Thornel P-55S, Cytec Engineered Materials, Greenville, SC, USA) into glass capillaries (GC120F-10, Clark Electromedical Instruments). Then the capillary was subsequently pulled using a vertical micro-pipette puller (PB-7, Narishige) into two similar part with short protruding carbon fiber at the tapered end. To limit the electroactive area to the very carbon fiber tip, poly-oxyphenylene polymer was deposited onto the protruding carbon fiber as an insulating layer. After that, a drop of mercury was back-filled into the capillary to achieve electrical contact. The CFE was cut at an appropriate length and beveled at an angle of 45°C for 5 min on a diamond particle whetstone micro-grinder (EG-4, Narishige Co., London, UK). Only the electrodes showing very stable amperometric baselines were used for single cell detection.

2.4. Amperometry at single cell

Electrochemical recording of exocytosis from single PC12 cells were performed on an inverted microscope (Observer D1, Carl Zeiss AG) inside Faraday cage. Before the experiment, the cells were rinsed three times with PBS and were maintained in HEPES physiological saline (150 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM Glucose, 10 mM HEPES and 2 mM $CaCl_2$) throughout the experiment at room temperature. The CFE was positioned by a micro-manipulator (Model MHW-103, Narishige Co., London, UK) in contact with the membrane of an independent PC12 cell. A glass micro-capillary containing stimulation solution (55 mM NaCl, 100 mM KCl, 1.2 mM $MgCl_2$, 5 mM Glucose, 10 mM HEPES and 2 mM $CaCl_2$) was positioned by another micro-manipulator at about 20 μm away from the cell (Fig. S1). All the amperograms were collected from individual cell stimulated by 60s' K^+ injection. Each cell was detected only once. During each experiment, the electrode was held at a constant potential vs. a Ag/AgCl reference electrode using a commercially available picopotentiostat (model AMU-130, Radiometer Analytical Instruments, Copenhagen, Denmark), for which the adjustable time-response was 1 ms (1 kHz low pass filter). In the following all potentials reported refer to the Ag/AgCl reference electrode. The output was digitized at 40 kHz and the current was recorded as a function of time (Powerlab 4SP A/D converter and software Chart 5.0, ADinstruments, Colorado Springs, CO, USA) with no subsequent digital filtering. In order to avoid surface fouling, the CFE was repolished between successive detections. Each amperometric trace recorded during cell secretion was visually inspected. Current spikes at least 3 times higher than the baseline fluctuation (0.3 pA) were identified as exocytotic events.

3. Results and discussion

Internalization of FFN102 by PC12 cells was achieved through incubation in culture medium supplemented with FFN102 (20 μM, 1 h), in line with conditions reported by Lee et al. [10]. Fluorescence imaging of pre-incubated cells revealed blurry fluorescent vesicles due to high fluorescent background as presented in Fig. 1. In contrast, cells exhibited no fluorescence when the cultured medium was not supplemented with FFN102. Although vesicles were not clearly defined, the total fluorescence intensity of the cells was lower after secretion,

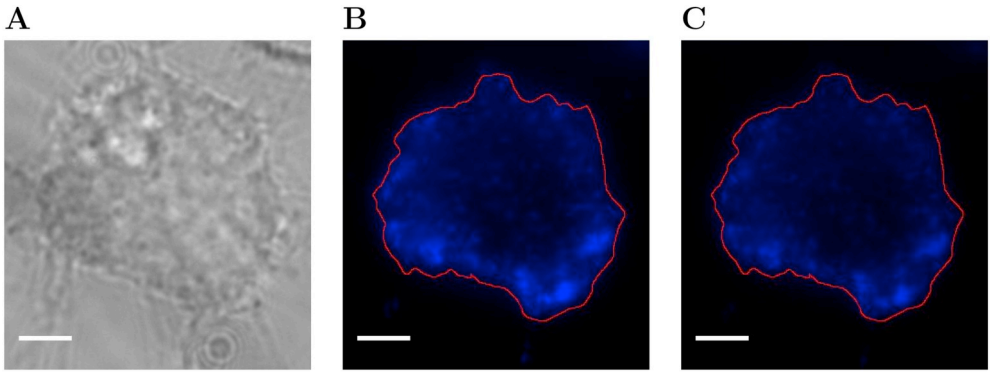

Fig. 1. A: Bright field image of a PC12 cell pre-incubated with FFN102 (20 µM, 1 h). B,C: Fluorescence background substracted image of the same PC12 cell before secretion (B) and after secretion (C) (filter set 49, Zeiss). The total fluorescence intensity within the region of interest (red line) decreased by 22% after secretion (ImageJ software [18]). Scale bar: 2 µm.

consistent with the release of FFN102 and the oxidation induced quenching at the detection electrode. The uptake of FFN102 in the vesicles of PC12 cells was further characterized using Amperometry at carbon fiber microelectrode (CFE).

The release of neurotransmitters from PC12 cells under stimulation was selectively monitored in order to detect either solely dopamine or dopamine and FFN102 altogether. Amperometric traces were recorded with the CFE potential poised at 650 mV or 900 mV respectively, according to the reported values allowing fast oxidation of dopamine and FFN102 at CFE [15,19,20]. Amperometric detection of exocytosis was performed with both FFN102 pre-incubated cells and control cells. More than 300 exocytic events at 16 cells were analyzed under each experimental conditions. Amperometric spikes were analyzed by a lab-made software to yield the maximum oxidation current I_{max} (pA), the width at half height $t_{1/2}$ (ms) and the total electrical charge Q (fC) (Fig. 2). The results are presented in Table 1. Among the analyzed amperometric spikes, 20% exhibited a so called "prespike foot" related to the release of transmitters through the initial fusion pore [21–24] (Fig. 2).

As shown in Table 1, the average detected charge, when selectively detecting the dopamine (with the CFE potential held at 650 mV), was lower at FFN102 treated cells compared to control cells: 55.9 ± 1.7 fC vs. 63.6 ± 1.8 fC respectively. Accordingly, $t_{1/2}$ and I_{max}, related to the width and the height of the peak were lowered for FFN102 treated cells. Conversely, oxidizing simultaneously dopamine and FFN102 at the CFE, led to similar values of the average detected charge at control cells (potential held at 650 mV) 67.0 ± 2.1 fC vs. pre-incubated cells (potential held at 900 mV) 67.1 ± 2.2 fC. Moreover, the median of the charge detected for dopamine alone (CFE potential held at 650 mV) at treated cells was significantly different from any of the three other detected charges as shown in Fig. 3.A (p-values $< 10^{-5}$, Mann-Whitney-Wilcoxon test). The median of the detected charge at control cells (CFE potential held at 650 mV or 900 mv) and at treated cells with CFE held at 900 mV were not significantly different between each other (p-values > 0.3, Mann-Whitney-Wilcoxon test).

The uptake of FFN102 therefore occured in the secretory vesicles of the PC12 cells and thus led to the partial replacement of the endogenous dopamine. The replacement ratio of dopamine by FFN102 was thus be estimated as ca. 12% mol. Assuming a released fraction of 40%-65% of transmitters content per releasing vesicle [25,26], the uptake of FFN102 led to the internalization of 37×10^3 - 60×10^3 molecules per vesicle. The concentration of the uptaken FFN102 was estimated considering a vesicle diameter of ca. 160–200 nm [25,27,28]. The corresponding concentration, 15–45 mM was three orders of magnitude higher than the incubation concentration.

The upload of FFN102 did not modify the secretion features of PC12 cells otherwise. Indeed, the cumulative numbers of exocytic events were similar for treated and control cells (see Fig. S2). The slight increase in cumulative numbers of exocytic events for treated cells was likely due to the renew of culture medium during the pre-incubation step. In addition, in all cases 20% of spikes exhibited a foot current (see Figs. S3 and S4).

The prespike feet with current values I_{foot} from 10% up to 50% of the maximum peak current were selected for analysis as depicted in Fig. 2. The observed frequency ratio of 20% of spikes exhibiting a foot is lower than the 30% similar value previously reported at PC12 cells [30] and at chromaffin cells [31]. It was presumably related to the different shape of the typical spikes recorded at our PC12 cells compared to the shape reported for some other PC12 cell lines [30,32]. Indeed, the

Table 1
Main features of the detected spikes at control cells "none" and pre-incubated cells "20 µM, 1h"; CFE potential E_{CFE}, number of analyzed spikes n, detected charge Q, peak current I_{max}, and duration at half peak height $t_{1/2}$. Secretion was monitored at 16 single cells in each experimental condition. Reported values are mean ± sem.

FFN102	E_{CFE} (mV)	n	Q (fC)	I_{max} (pA)	$t_{1/2}$ (ms)
none	650	393	63.6 ± 1.8	2.45 ± 0.06	21.21 ± 0.21
20 µM, 1 h	650	434	55.9 ± 1.7	2.33 ± 0.06	18.91 ± 0.18
none	900	315	67.0 ± 2.1	2.82 ± 0.06	18.68 ± 0.44
20 µM, 1 h	900	379	67.1 ± 2.2	2.75 ± 0.10	19.78 ± 0.36

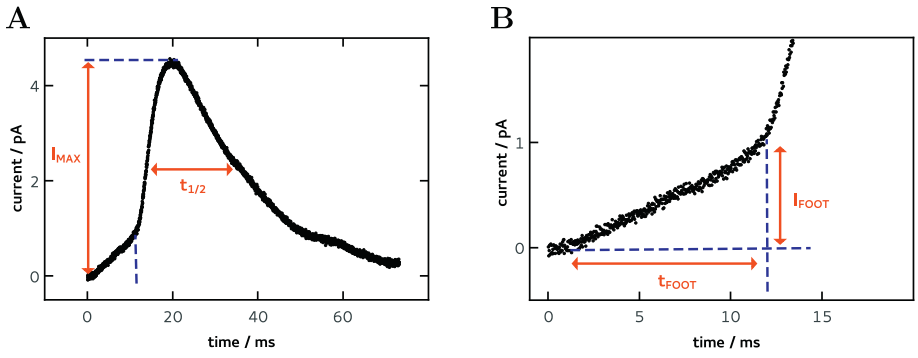

Fig. 2. A: typical recorded spike displaying a prespike foot with the representation of maximum oxidation current I_{max}, duration at half peak height $t_{1/2}$; the released charge Q being the area under the curve. B: enlarged view of the prespike foot with the representation of the foot current I_{foot} manually determined through time t_{foot}, Q_{foot} the integrated current during t_{foot}.

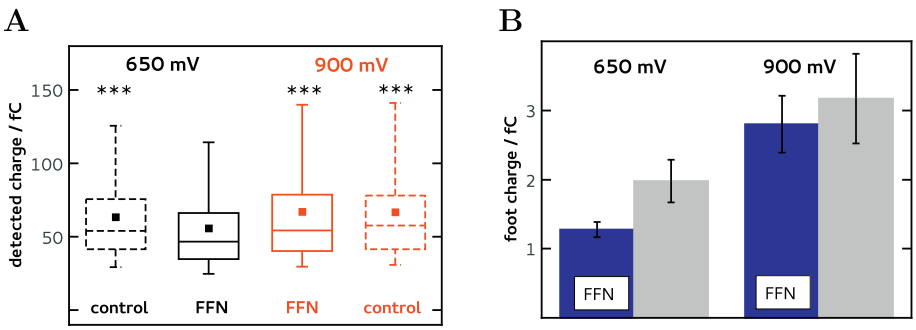

Fig. 3. A: Features of the detected charge distributions during selective detection of dopamine (650 mV) and during detection of dopamine and FFN102 (900 mV) at treated cells (solid line) and control cells (dashed line). Horizontal lines correspond to the three first quartiles, whiskers span the 5%-95% range and plain dot indicates the mean value. ⋆⋆⋆ $p < 10^{-5}$; p-values obtained from Mann-Whitney-Wilcoxon test (R software [29]). B: Average detected charge in spikes feet. Error bars are sem.

Table 2
Pre-incubation conditions "FFN102", detected released charge Q, detected foot charge Q_{foot}. CFE potential was held at 650 mV. Secretion was monitored at 16 single cells in each experimental condition. Reported values are mean ± sem.

FFN102	Q (fC)	Q_{foot} (fC)
none	78.1 ± 3.8	1.97 ± 0.31
20 µM, 1 h	70.9 ± 2.9	1.28 ± 0.11

spikes measured at our PC12 cells exhibited lower peak current and larger duration time compared to the one described in ref. [32]. Consequently, the average foot current is about 2–3 fold the amplitude of the noise only which could explain lower occurrence of detected pre-spike feet (see Fig. S3–S4).

Expectedly, when only dopamine was oxidized at the CFE, the feet charges were lower for treated cells than for control cells (Fig. 3.B). On the contrary, feet charges values were similar when both dopamine and FFN102 were detected (Fig. 3B). Interestingly, when cells were pre-incubated with FFN102, FFN102 had an enhanced contribution to the released charge through the fusion pore. Indeed, FFN102 replaced ca. 35% of the dopamine during foot duration whereas this ratio fell to 9% during the whole spike duration. The values of the averaged detected charges related to spikes exhibiting a foot are presented in Table 2. The averaged released charge was significantly higher for spikes featuring a foot (78.1 ± 3.8fC) than for spikes without foot (63.6 ± 1.8fC). This trend has already been reported for L-DOPA treated chromaffin cells [31] and PC12 cells [30]. The loading of FFN102 observed in secretory vesicles of PC12 cells was thus analog to the reported L-DOPA one. This uptake was therefore consistent with the enrichment of a fast diffusion compartment in the vesicle (so called "halo") [24,33] and with a recently introduced model relying on the coexistence of condensed and uncondensed chromogranin within secretory granules [34]. Interestingly, a similar interference of β-blockers with the loading of dopamine in chromaffin cells vesicles was suggested to rationalize some of their effects [35].

4. Conclusions

We have demonstrated the suitability of FFN102 to be used as a probe for bioanalytical studies at PC12 cells, a widely used secretory cell model. Interestingly, the fact that dopamine and FFN102 are oxidized at different potentials was used to evaluate the proportion of both species in treated cells. The uptake of FFN102 led to the partial replacement of dopamine in secretory vesicles. Furthermore, the FFN102 was over released through the initial fusion pore suggesting that its storage mostly occured in fast diffusion compartment of the vesicles. The partial replacement of dopamine could be used to monitor exocytic events through electrochemical detection of dopamine and fluorescence detection of FFN102. This approach could be of interest to overcome the possible shortcoming of the high oxidation potential of FFN102. Moreover, the FFN102 enrichment of transmitters released through the fusion pore could enhance the efficiency of FFN102 to report fusion pore opening when used as a fluorescent probe.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2018.11.001.

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