AFM and neurodegenerative diseases (part II): Correlating Atomic Force Microscopy (AFM) and Fluorescence Microscopy to study the interaction between Dopamine and the D1-Receptor in SH-SY5Y Cells

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In the present study, the BioScope™ II Atomic Force Microscope has been integrated with epifluorescence for the purpose of studying the interaction between dopamine and its membrane receptor at the cell membrane of living cells.

INTRODUCTION

In addition to its high resolution imaging capabilities, atomic force microscopy (AFM) has emerged as a powerful tool for measuring both the nanomechanical properties and interaction forces of biomolecular complexes. While the majority of these types of AFM studies have been conducted on isolated molecules[1], for true biological relevance these investigations should ideally be conducted on live cell systems.[2-4]

In addition, it now appears essential to be able to couple AFM with other techniques like optical microscopy in order to simultaneously collect both types of data. So far, very few studies are based on the combination of AFM and inverted optical microscopy.[5-7]

In the present study we investigated the potential application of force measurements in monitoring the response and binding properties of dopamine D1 receptor upon stimulation with dopamine. We used our fully integrated BioScope II AFM and epifluorescence system to correlate fluorescence imaging and AFM force measurements.

Dopamine (DA, 4-(2-aminoethyl) benzene-1,2-diol) is a major neurotransmitter belonging to the catecholamine family, based on an aromatic amino-acid called tyrosine, and is the precursor of two main hormones: adrenalin and noradrenalin.

In the peripheral nervous system, the main role of dopamine is to modulate cardiovascular functions as an analeptic, hormone turnover, renal function, vascular flow and gastrointestinal motility. In the central nervous system (CNS), dopamine is involved in the control of locomotor functions, cognition, emotion, food intake and endocrine regulation.[8,9] Dysfunction of dopaminergic neurotransmission in the CNS is linked to a variety of neuropsychiatric disorders, including Tourette's syndrome, Parkinson's disease, schizophrenia, paranoia, and Attention-deficit hyperactivity disorder (ADHD). Dopamine receptors are classified from D1 to D5 of which D1 and D2 receptors make up the largest proportion (for a detailed description of receptors see Ref. 10). For treatment of these diseases the identification of dopaminergic drugs devoid of side effects is one of the biggest challenges in neuronal research and drug discovery.

In this particular study, we used a YFP-labeled transmembrane D1 receptor to investigate the specificity of the interaction between this receptor and a dopamine-modified AFM tip, using both the force spectroscopy and the optical imaging features of our integrated tool.

SAMPLE PREPARATION:

SH-SY5Y cells were transfected with YFP-tagged Dopamine D1 receptor (DRD1-EYFP). Transfection was performed by nucleofection (Nucelofector, AMAXA) using a cell suspension (10^6 cells/ml), 4 µg plasmids DNA, and 100 µl transfection buffer

(AMAXA). Subsequently, cells were seeded on sterile cover slips (18x18 mm) in 6-well plates. 48 h after transfection adherent cells expressing the DRD1-EYFP were stimulated with 10–50 μM Dopamine hydrochloride.

Brightfield (BF), DIC and epifluorescence images were acquired on a Zeiss Axio Observer inverted microscope equipped with an AxioCam MRC camera and the AxioVision software. All AFM images were recorded on a Veeco BioScope™ II that has been fully integrated with the optical microscope, and using DNP/ MSCT cantilevers.

All experiments were performed in contact and TappingMode™ using PBS buffer and the softest DNP- and MSCT-type cantilevers (nominal spring constant 0.06 N/m and 0.01 N/m, respectively.). Dopamine-functionalized cantilevers were prepared as previously described.[11]

Briefly, a polyethylene-glycol (PEG) derivative, having an amino-reactive end and a thiol-reactive end, was used as a linker and as an inert back-filling molecule so that only dopamine could contribute to the observed binding interactions. Spring constants were calculated in fluid on a stiff support (glass bottom of the Petri dish) using the thermal tune option of the Veeco NanoScope® Controller and deflection sensitivities were manually updated using the integrated PicoForce software capabilities. A total of 3072 force curves were recorded in force volume mode, at scan rate between 3 and 3.5 Hz, and with a retract delay of 10 ms. The scan area was 2x2 μm.

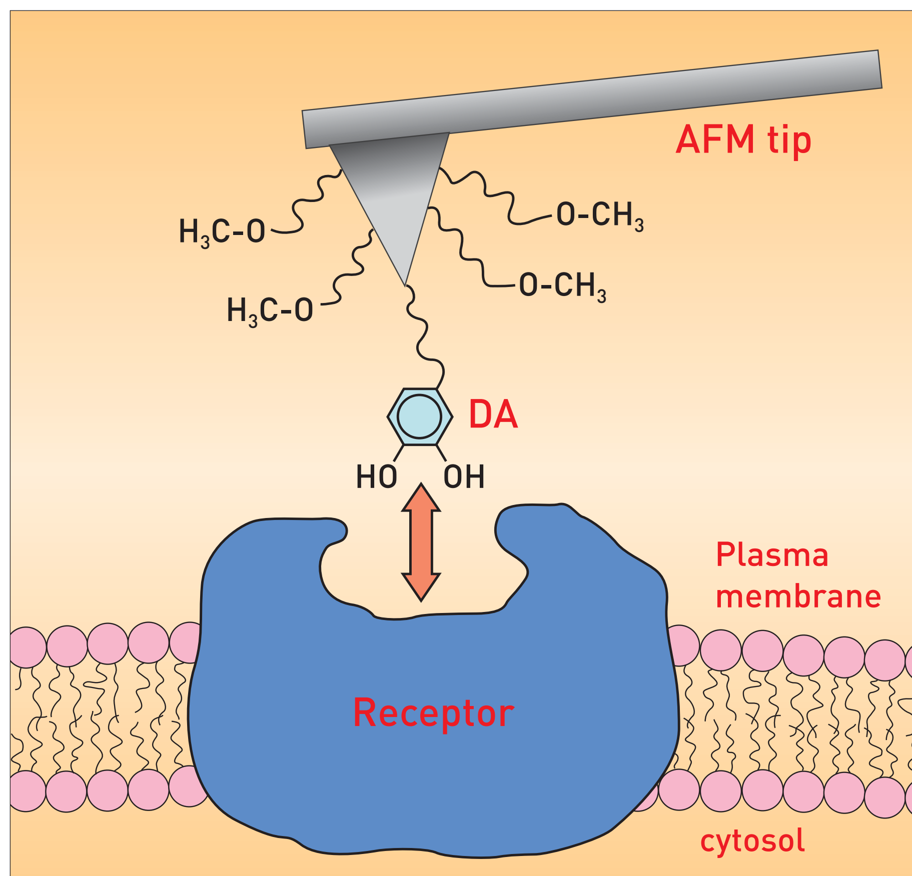

Figure 1: Studying ligand-receptor interactions on a living cell. The AFM tip has been functionalized in such a way that the chemical moieties of dopamine (DA) involved in the interaction with its D1-receptor are preserved. In addition, the length of the spacer minimizes the effect of the tip on the interaction itself.

USING AFM TO TRACK DOPAMINE BINDING TO MEMBRANE-BOUND D1 RECEPTORS

The overexpressed YFP-tagged D1 receptors localize mainly in the plasma membrane and show a random distribution in neuronal SH-SY5Y cells. Based on fluorescence data the receptors internalize into the cytoplasm upon stimulation with dopamine. In order to support this hypothesis and to test the potential application of AFM, we used the BioScope II force spectroscopy capabilities to track binding of DA to membrane-bound D1 receptors and their subsequent internalization.

First, DNP cantilevers were functionalized with a dopamine analog as illustrated in Figure 1. In the next step live cells were exposed to several DA concentrations from 10 to 50 μM in order to stimulate the D1 membrane receptors.

Both epifluorescence images and time-lapse movies were recorded to detect changes in the fluorescent signal of D1 receptor distribution and consequently, to investigate the internalization and binding properties of the YFP-tagged dopamine D1-receptor.

Figure 2A shows the experiment at t_0: at this time point, the dopamine has not been applied and thus, D1 receptors are not stimulated. As a result, the YFP-tagged D1 receptors remain membrane-bound revealing a homogeneous fluorescent staining across the cell surface. A significant change in the fluorescent distribution is achieved upon stimulation of the D1-receptor by DA. Figure 2B shows a typical example of the images recorded at t_{10min}. For all DA concentrations, no membrane fluorescence was detected after 10 minutes incubation.

Instead, multiple small intracellular fluorescent vesicles variable in size and brightness were observed indicating possible receptor internalization (Figure 2B). This observation points to an active binding of the ligand DA to the D1 receptor in the SH-SY5Y cell model. Note that weak fluorescence signals required us to use long exposure times to obtain the fluorescence images. In order to avoid possible bleaching effects we closed the fluorescence shutter during the application of DA and opened it just before acquiring the image.

In addition to fluorescence imaging, DA – D1-receptor unbinding forces were recorded using AFM force volume mode on living cells at different time points in

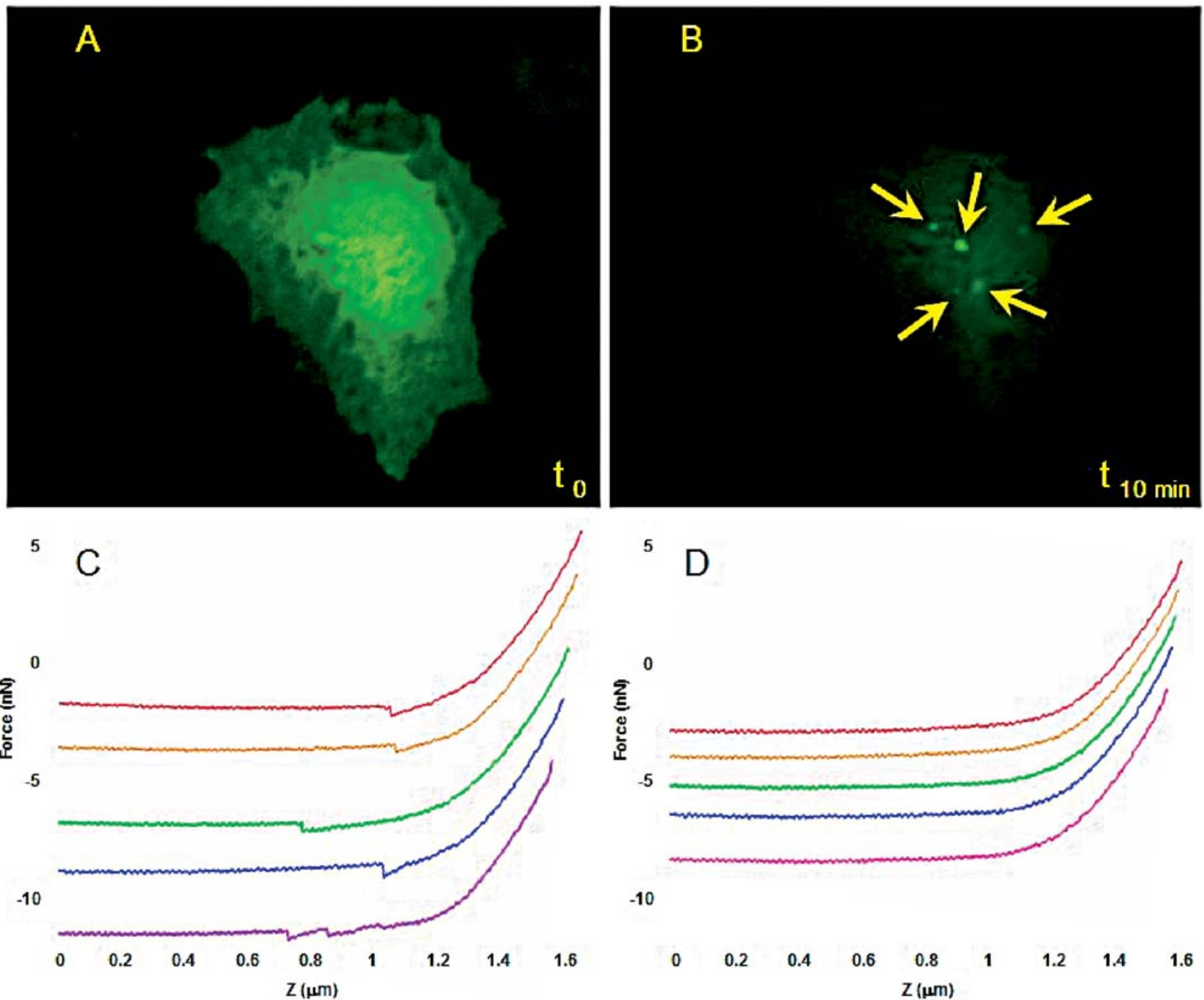

Figure 2: Correlating fluorescence imaging and AFM force measurements. The AFM tip was tagged with a DA analog to follow the location of YFP-tagged D1-receptors before and after applying free DA. Before stimulation by DA, receptors are cell membrane-bound and thus, fluorescence is distributed all over the cell surface (A) and force curves exhibit a high percentage of specific unbinding events (C). 10 minutes after DA application, all the fluorescence is concentrated into small dots (B) and no specific events are recorded in force volume proving that all the receptors have been internalized into the cytosol.

the presence and absence of dopamine. Force volume imaging of a single cell with a DA-functionalized tip at time point t_0 (no stimulation of D1-receptors) resulted in a significant portion (13.12%) of force curves exhibiting a specific unbinding event (Figure 2C).

In parallel to optical observations, the force measurements were also performed at time point t_{10min} (after stimulation of D1-receptors by DA). As shown in Figure 2D, all recorded force curves exhibited no specific unbinding event, thus supporting our observations by fluorescence imaging of the internalization of the D1-receptors into the cytosol. We did not find this phenomenon to be reversible even after 1 hour of DA stimulation. Comparison of images and time-lapse movies confirmed that the observed change of fluorescence pattern is a direct consequence of the internalization process.

RESULTS

In our studies, DA – DI-receptor interactions exhibited a single unbinding force centered around 223 ± 82 pN. Interestingly, the mean peak value was stable when measured at the edge of the cell, whereas strongest variations and higher values were observed for measurements taken near the cell nucleus. To date, the interaction mechanism between dopamine and its receptor is not fully understood.

Studies report the pharmaceutical characteristics of dopamine, as well as many of its agonists and antagonists, to its potential receptors.[12-15] Depending on the receptor type, the dissociation constants were found to be between 880 and 2980 nM.[12]

Dynamic force spectroscopy can also be used for determination of kinetic parameters. In our working setup, all AFM scans were performed under the same conditions, but it is also possible to vary the scanning parameters and plot the unbinding force as a function of the loading rate. Curve progression can provide information as to whether the if kinetics of the unbinding process is more dependent on the inner or

outer barrier of the energy landscape. The kinetic off-rate constant (K_{off}) of dissociation can also be determined.[16-19]

By essentially, plotting the adhesion force as a function of the logarithm of the loading rate during the retraction, while keeping constant the interaction time and the approach speed, will reveal the length scale of the energy barrier. Extraoplation of the curve at force zero will give K_{off}.

On the other hand, plotting the adhesion frequency as a function of the interaction time while keeping constant the approach and the retraction speed will give the interaction time needed for half-maximal probability of binding. Finally, knowing the association rate constant (K_{on}), the equilibrium constant can be determined as follows: $K_D = K_{off}/K_{on}$

CONCLUSION

The present investigation demonstrates the potential of combined AFM and fluorescence microscopy to study the presence and binding properties of dopamine D1-receptors on the surface of live cells. In this two-part series of Application Notes on the use of AFM in the study of neurodegenerative diseases (See AN117: Part I) experiments focused on the integration of two major techniques used in life science applications. Combining optical microscopy techniques with the BioScope II AFM allows:

1) 3D identification of molecules of interest by BF, DIC, fluorescence and AFM height images.

2) Investigation of the physical properties of the target molecule through optical imaging and topographic, friction, viscoelastic, and adhesion data.

3) Real-time observation of a cellular signaling event at the submicron scale.

4) Measurement of the specific unbinding force between a ligand and its receptor, and to a further extent, determination of their kinetic parameters. This last option opens huge possibilities in the field of pharmacology, especially drug discovery.

By combining optical microscopy with the AFM capabilities of the BioScope II, this not only provides users with the benefits of both techniques in a single experiment, it also offers easy access to the sample and a high flexibility of manipulation. We believe that these studies provide convincing results as to the value of such multimodal instrumentation and will help speed advances in neurology research.

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