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Assessment of *in vitro* dopamine-neuroblastoma cell interactions with a bioelectric biosensor: perspective for a novel *in vitro* functional assay for dopamine agonist/antagonist activity

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ABSTRACT

Current receptor-binding assays for dopamine do not measure the *in vitro* whole cellular response against dopamine or potential agonist/antagonist molecules. We herewith report the development of a novel functional assay concept for studying the *in vitro* interaction of the neurotransmitter dopamine with neural cells bearing dopamine receptors. The concept is based on the ultra-rapid measurement of changes in the electric properties of cultured N2a mouse neuroblastoma cells (corresponding to cumulative changes of the cell membrane potential). A close relationship between cumulative cell membrane potential and dopamine concentration was observed. Membrane depolarization was observed at nanomolar dopamine concentrations, while hyperpolarization was associated with micromolar ones. Treatment with the dopamine D2-receptor antagonist eticlopride resulted to a concentration-dependent membrane depolarization. Treatment with sodium chloride caused considerable weakening of the dopamine-associated hyperpolarization effect. The observed bioelectric response to dopamine was highly inversely correlated with the pattern of dopamine release-uptake balance by N2a cells, as determined with cyclic voltammetry. The bioelectric approach was also used to evaluate the dopaminergic activity of chaste tree (*Vitex agnus-castus*) extracts. The novel assay concept offers promising perspectives for the development of advanced companion diagnostics system for the high throughput, fast functional characterization of neurotransmitter agonists and antagonists.

1. Introduction

Dopamine (DA) signaling is one of the most important aspects of brain function. In particular, the pharmacological modification of the responsiveness of the different dopamine receptors (D1-D5) and their subtypes is the basis of the treatment, as well as the side effects of major diseases and pathological conditions, such as schizophrenia, Parkinson's disease, Tourette syndrome, drug addiction, and hyperprolactinemia [1]. In addition to receptor-affinity studies, functional assays for testing the activity of drugs with agonistic/antagonistic dopaminergic activity are invaluable to neuropharmacology research. Even though they often utilize only a small percentage of the total receptor population in the sample biological system, they offer increased and more accurate information about the efficacy and potency of the assayed drugs, especially at very low agonist concentrations [2].

Over the last decade, mammalian cell-based assays and biosensors have grown considerably in significance in clinical analytical science, offering the combined advantage of high-sensitivity and non-invasive or minimally invasive monitoring. Even more important is their multipurpose catalytic capacity, enabling cellular biorecognition elements to provide information about the actual effects of target analytes, even if these effects represent the synergistic or cumulative response of the interaction of said analyte with different receptor subtypes [3,4]. In addition, assay principles based on measuring cellular bioelectric properties (such as impedance or membrane potential) are quite popular, due to their speed, quantification and relative ease of use. Among these techniques, the Bioelectric Recognition Assay (BERA) is designed in order to rapidly measure changes in the membrane potential of a cell population and has been used in numerous applications, including, quite recently, the non-invasive determination of superoxide levels during the *in vitro* differentiation of PC12 cells [5]. Although there exist several electrophysiological assays like the patch-clamp technique [6], sharp electrode technique, single-unit recording [7], SSM-based method [8] with unique application characteristics, the BERA method is advantageous in respect of offering ultra-high speed (an assay can be completed in less than 3 min), high sensitivity and reproducibility, low cost and is quite user-friendly, therefore allowing

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its application in high throughput tests. So far and regarding neural cells, the BERA method was used for the detection of neuroactive toxic substances, such as pesticides [9–11].

In the present study we report a novel methodological concept for the development of functional assays for the *in vitro* interaction of DA with N2a mouse neuroblastoma cells utilizing the BERA principle. The novel approach is characterized by considerable speed (3 min), sensitivity (measuring the response of cells against DA concentrations as low as 1 nM) and reproducibility, while it allows for an accurate monitoring of DA release-uptake balance pattern by cultured cells, as determined in parallel with cyclic voltametry. The effects of the selective dopamine D2-like receptor antagonist eticlopride and sodium ions as a non-selective antagonist were also assessed using this novel approach.

Finally, the novel approach was used for the assessment of the dopaminergic activity of chaste tree (*Vitex agnus-castus*) extracts. Several reports have shown the considerable dopaminergic effects of this plant species, traditionally used for treating dopamine-related pathological conditions, such as cyclical mastalgia inhibiting the release of overage prolactin [12], premenstrual mastodynia, cycle irregularities [13]. The fruit of *V. agnus-castus* has been used to treat menopausal symptoms and premenstrual syndrome in which some symptoms are irritability, mood alteration, headache and bloating [14,15]. The extracts of *V. agnus-castus* showed significant decrease of the above symptoms [15] and have been shown to act as DA agonists [14,16].

The perspectives for developing new types of cell-based functional assays for application in high throughput neuropharmacology screening are discussed.

2. Experimental

2.1. Chemicals

Murine neuroblastoma (N2a) cell cultures were originally provided from LGC Promochem (UK) and subcultured in Dulbecco's medium with 10% fetal bovine serum (FBS), 1U μg^{-1} antibiotics (penicillin/streptomycin) and 2 mM L-glutamine which were provided from Invitrogen (CA, USA). All other reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany).

2.2. Cell membrane potential measurements: biosensor set-up

Changes of the cell membrane potential were measured using a customized, multichannel potentiometer (Uniscan, Buxton, UK), allowing for up to eight simultaneous measurements from respective eight carbon screen-printed electrodes on a disposable sensor strip (DropSens, Asturias, Spain) [19] (Fig. 1A). Reference electrodes were made of Ag/AgCl.

Before each assay, cells were detached from the culture and concentrated by centrifugation (2 min, 1200 rpm, 25 °C). After centrifugation cells were resuspended in DMEM medium. During each assay, cells in suspension were placed on the top of each of the eight carbon screen-printed electrodes contained in the sensor strip (45 μL≈50×10^3 cells) with the help of a multichannel automatic pipette (Fig. 1B). Next, 5 μL of sample (DA solution) were added (Fig. 1 C).

The response of the cells to the different samples was recorded as a time-series of potentiometric measurements (in Volts) (Fig. 1D). At each different DA concentration, the final value of the potentiometric cell response was calculated by subtracting the recorder response against DA alone (cell-free measurement) from the observed cell-mediated response. The duration of each measurement was 180 s and 360 values/sample were recorded at a sampling rate of 2 Hz. Modifying the sampling rate (from 2 to 20, 50 or 100 Hz) did not affect the measurements.

For investigating the effects of the selective D2-like receptor antagonist eticlopride [17], cells were treated with different eticlopride concentrations (same concentration range to DA). DA and eticlopride solutions in double distilled water were prepared freshly on the day of each assay. It has been previously reported [18] that sodium chloride is able to fully convert D2-like receptors from a high-affinity to a low-affinity state for DA. Therefore, in order to investigate the effect of sodium ions as a non-selective antagonist of D2-like receptors, cells were also first incubated in 120 mM NaCl for one hour and then transferred to biosensor for treatment with DA. In order to exclude the effect of chloride ions, in a separate experiment cells were incubated in 120 mM choline chloride prior to their transfer to the biosensor.

2.3. Estimation of DA release-uptake balance

The release-uptake balance pattern by cultured N2a cells was determined with cyclic voltammetry using a sweep rate of 100 mV s^{-1} and a scan potential between −1,0 V and +1,0 V. Reference electrodes were made of Ag/AgCl. Briefly, N2a cells (at a density of 10^6 cells) were incubated with 50 μL of medium (DMEM) with different DA concentrations for three minutes (in eppendorf tubes). At the end of incubation the supernatant (50 μL DMEM without cells) was collected, after centrifugation, from each eppendorf tube and assayed for DA. In each voltammogram, the magnitude of current change (in μA), corresponding to the oxidation peak observed at +0,37 V (Fig. 3A), was used for the indirect determination of DA concentration. The biosensor was calibrated with a series of DA solutions (0–1000 μM). Each calibration test was carried out three minutes after preparing the fresh DA solutions. Therefore, calibration data could be comparable with the results of the release-uptake balance measurements.

2.4. Estimation of dopaminergic activity of V. agnus-castus

The DA extraction of *V. agnus-castus* was achieved by the homogenization of one gr immature fruit in 10 mL 70% methanol and the homogenate was inserted in an ultrasonic bath for 20 min. Then, the sample was filtered through Whatman filter paper and sample dilutions were made to prepare 1% and 10% (v/v) extract concentrations in water. The dopaminergic activity was estimated by measuring changes of the cell membrane potential, after adding 5 μL of sample to N2a cells having or not previously blocked the D2 receptors using 5 μL of eticlopride solution as described above (Section 2.2).

2.5. Experimental design

Experiments were set up in a completely randomized design. A set of eight parallel, simultaneous measurements was recorded for each individual sample and each experiment was repeated three times.

3. Results and discussion

3.1. N2a cells response to DA concentrations

The results of the biosensor-based potentiometric assay demonstrated two distinct patterns of response depending on the range of DA concentrations: (a) a concentration-dependent increase of the cumulative membrane potential (bioelectric potential) of cultured N2a cells in the range of 0–100 nM, indicating membrane depolarization (Fig. 2A) and (b) a gradual reduction of the cumulative membrane potential at the range of 1–1000 μM, indicating membrane hyperpolarization (Fig. 2B).

It has been previously reported that different DA receptors have widely different affinities for DA as well as different, frequently opposite effects: for example, D1-like receptors stimulate cAMP accumulation, leading to membrane depolarization. On the other hand, activation of D2-like receptors is associated with inhibition of cAMP accumulation and the increase of outward potassium currents, therefore leading to cell membrane hyperpolarization compared to control

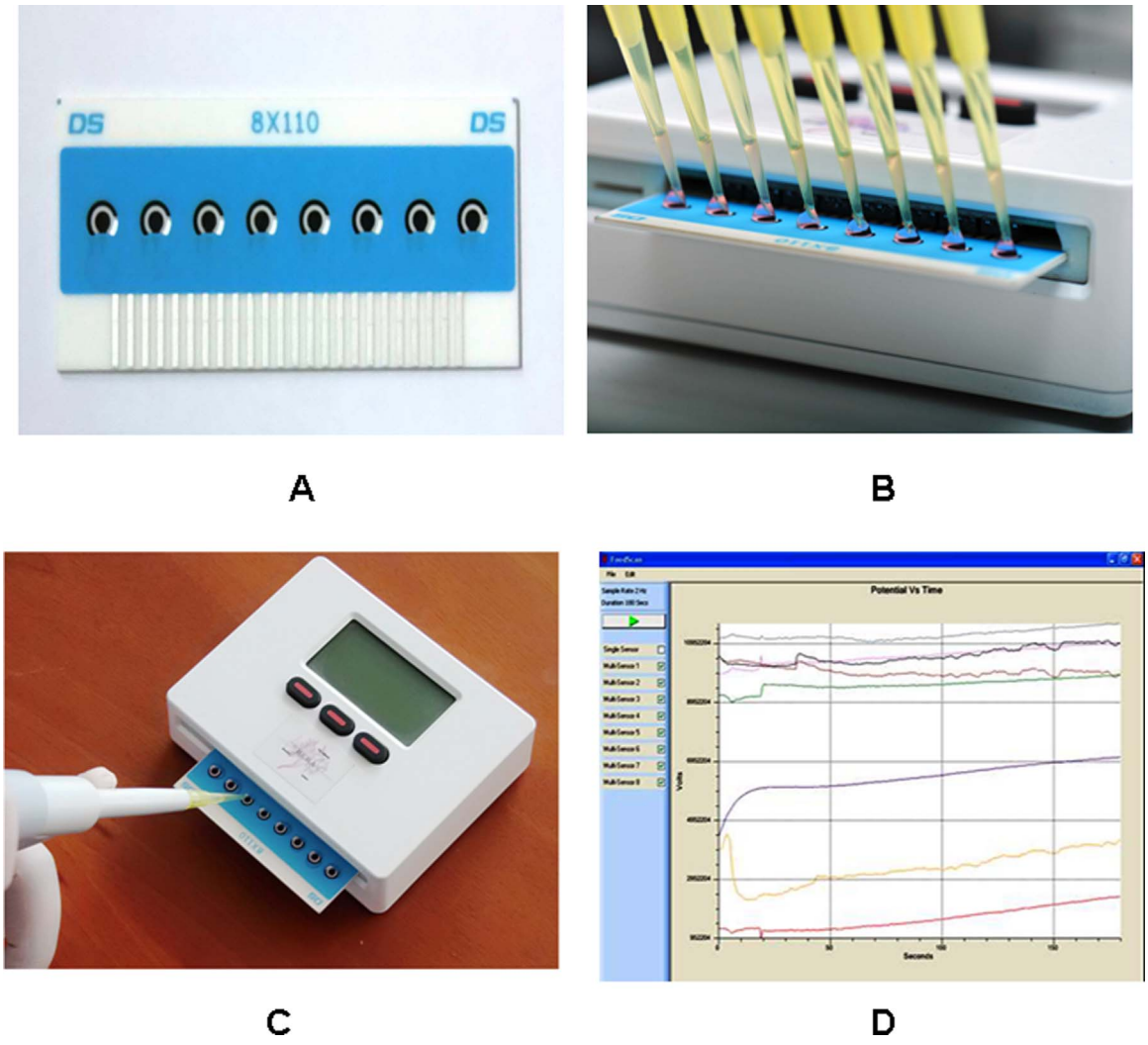

Fig. 1. (A) The eight-channel disposable sensor, (B) Addition of cell suspension on the top of each of the eight carbon screen-printed electrodes, (C) Addition of sample (DA solution) in the cell suspension, (D) Display of real-time measurements using the dedicated software.

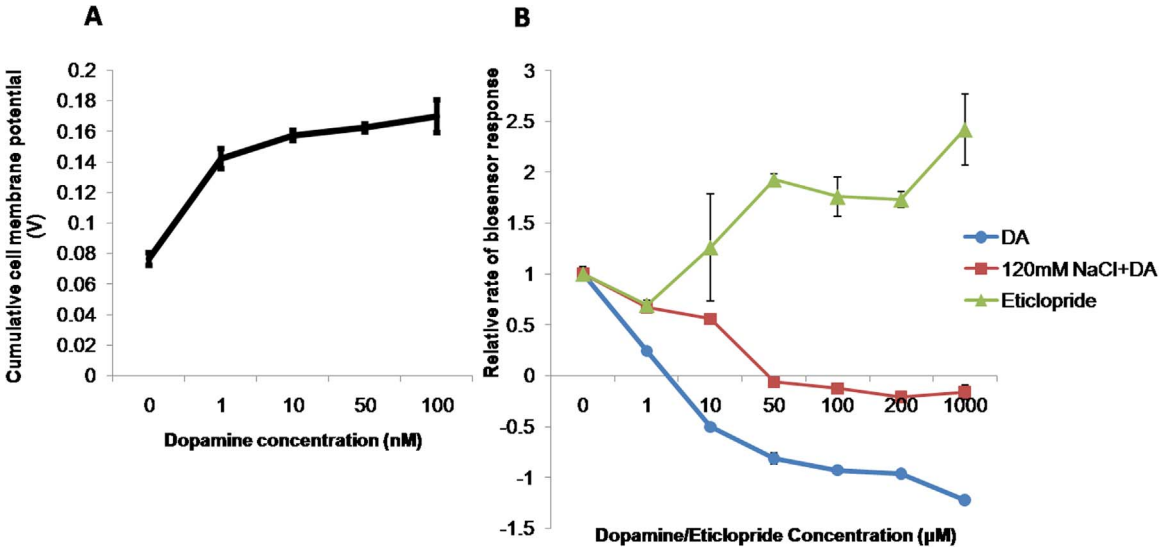

Fig. 2. Biosensor responses to different treatments. (A) N2a cells response to increasing DA concentrations at the *nanomolar* range (1–100 nM). (B) N2a cells response to increasing *micromolar* (1–1000 μM) DA concentrations (*circles*), after treatment with eticlopride (*triangles*) and after incubation of the N2a cells in 120 mM NaCl (*squares*). Results are shown relative to control (no treatment). Each data point represents the average of three individual experiments, each with eight measurements per sample.

(zero DA concentration) [20,21]. Therefore, the observed results may reflect a preferential DA binding (a) to D1-like receptors at low concentrations (nanomolar range) and (b) to presynaptic D2-like receptors at higher (micromolar) concentrations [22]. Trantham-Davidson et al. [23] have already demonstrated that DA concentration is a critical determinant of D1 *versus* D2 signaling in prefrontal cortex, with D1-like and D2-like receptors being activated by low (< 500 nM) and high (> 1μM) DA concentrations, respectively.

The novel bioelectric biosensor was also used to investigate changes of the cell membrane potential in response to eticlopride, a substituted benzamide which is a highly selective blocker of D2-like receptors compared to D1-like receptors, with Ki values of 0.09 nM *vs*. 10,000 respectively [18]. Eticlopride elicited a response quite opposite to DA, *i.e*. a concentration-dependent shift of the bioelectric potential towards positive values indicating membrane depolarization (Fig. 2B). Finally, incubation of cells in 120 mM NaCl, resulted in considerable weakening of the DA-associated hyperpolarization effect (observed as a shift towards positive values at all DA concentrations) (Fig. 2B). This was expected, since it has been previously shown that sodium ions reduce the affinity of D1 and D2-like receptors for DA and its agonists [19]. It should be mentioned that chloride ions did not have any effect at all: following incubation in choline chloride cells responded to DA in a pattern essentially identical to the control (r^2=0,98861) (analytical results not shown).

The observed results in each experiment were highly reproducible, with an average standard error of 4.4%.

3.2. DA release-uptake balance by N2a cells

The results of the cyclic voltametric DA (expressed as current values corresponding to different DA concentrations) assay revealed a concentration-dependent of release-uptake balance pattern of DA. This pattern was increased in an almost linear manner (r^2=0902) following exogenous DA application (calibration curve) in the range of 1–1000 μM (Fig. 3B). In this way, the pattern of DA release-uptake balance of N2a cells was inversely correlated with (r^2=−0.804) – or reflected at – the observed changes in cell membrane potential as recorded with the novel assay.

3.3. Assessment of the dopaminergic activity of V. agnus-castus extracts

The results of the evaluation of the dopaminergic activity of chaste tree (*V. agnus-castus*) extracts (Fig. 4) with the novel assay showed that, when applied at 1% extract concentration, samples did not elicit any significant cell response as assessed by differences between control (gray column) and eticlopride-treated (dark column) cells. The lack of any observed effects at the lowest extract concentration is probably due to the very low content of either DA or its agonists in the sample. On the contrary, the application of *V. agnus-castus* extracts at 10% concentration (*i.e.* tenfold) caused a significantly ($p < 0.05$) lower response in control compared to eticlopride-treated cells, therefore revealing the hyperpolarization of cell membrane due to the dopamine-agonistic effect of the extract. As expected, no significant response was observed when the dopamine D2-receptor antagonist eticlopride was added before the application of the 10% *V. agnus-castus* extract.

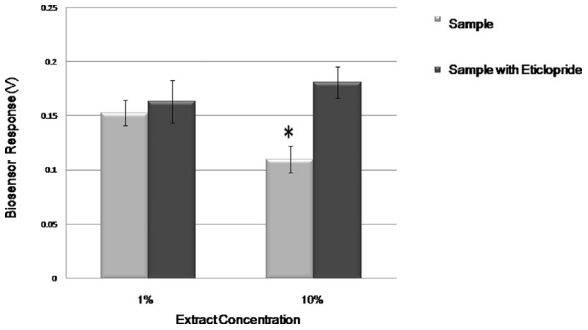

Fig. 4. Evaluation of the dopaminergic activity of immature fruit of *V.agnus-castus*by measuring the response of N2a cells to diluted fruit extracts. Prior to sample application, cells were treated (dark column) or not (gray column) with eticlopride. Data correspond to the mean ± SEM of three different experiments *p < 0.05.

4. Conclusions

The biosensor-based functional assay concept developed in the present study could be used as a basis for the development of a rapid, very sensitive, high throughput screening system of DA agonists and/or antagonists in order to assess their effect on whole cellular responses *in vitro*. Furthermore, as shown in the present study, the assay can be finely combined with a variety of advanced biosensor (mostly electrochemical) techniques for the rapid determination of DA concentration and the rate of its release-uptake balance after administration of the screened agonist or antagonist molecules [24], therefore increasing considerably the amount of information retrieved from a single *in vitro* experiment. As also demonstrated with the case of *V. agnus-castus* immature fruit extracts, the cell bioelectric approach could be a helpful tool for the rapid assessment of the dopaminergic potency of natural bioactive drugs and associated preparations, to be used in the framework of clinical trials and/or ethnobotanical studies.

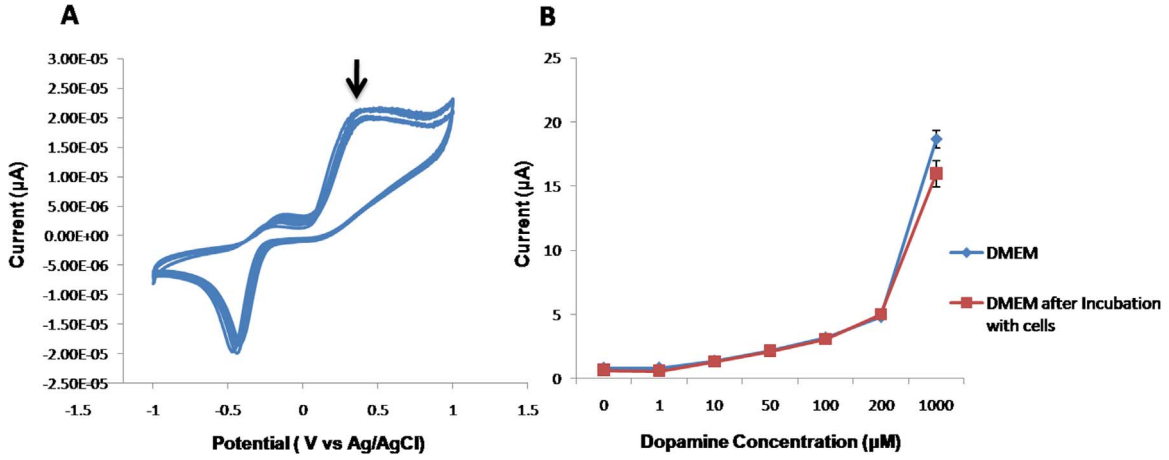

Fig. 3. (A) A set of five tandem cyclic voltammograms of DA (at 1000 μM). Arrow indicates the oxidative peak at 0.37 V (B). DA calibration curve (*circles)* and changes in DA release-uptake balance (expressed as current values) of N2a cells in response to increasing DA concentrations (*squares*). Each data point represents the average of three individual experiments, each with eight measurements per sample.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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