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Neurotoxicity screening of (illicit) drugs using novel methods for analysis of microelectrode array (MEA) recordings

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ABSTRACT

Annual prevalence of the use of common illicit drugs and new psychoactive substances (NPS) is high, despite the often limited knowledge on the health risks of these substances. Recently, cortical cultures grown on multi-well microelectrode arrays (mwMEAs) have been used for neurotoxicity screening of chemicals, pharmaceuticals, and toxins with a high sensitivity and specificity. However, the use of mwMEAs to investigate the effects of illicit drugs on neuronal activity is largely unexplored.

We therefore first characterised the cortical cultures using immunocytochemistry and show the presence of astrocytes, glutamatergic and GABAergic neurons. Neuronal activity is concentration-dependently affected following exposure to six neurotransmitters (glutamate, GABA, serotonin, dopamine, acetylcholine and nicotine). Most neurotransmitters inhibit neuronal activity, although glutamate and acetylcholine transiently increase activity at specific concentrations. These transient effects are not detected when activity is determined during the entire 30 min exposure window, potentially resulting in false-negative results. As expected, exposure to the $GABA_A$-receptor antagonist bicuculline increases neuronal activity. Exposure to a positive allosteric modulator of the $GABA_A$-receptor (diazepam) or to glutamate receptor antagonists (CNQX and MK-801) reduces neuronal activity. Further, we demonstrate that exposure to common drugs (3,4-methylenedioxymethamphetamine (MDMA) and amphetamine) and NPS (1-(3-chlorophenyl)piperazine (mCPP), 4-fluoroamphetamine (4-FA) and methoxetamine (MXE)) decreases neuronal activity. MXE most potently inhibits neuronal activity with an IC_{50} of 0.5 μM, whereas 4-FA is least potent with an IC_{50} of 113 μM.

Our data demonstrate the importance of analysing neuronal activity within different time windows during exposure to prevent false-negative results. We also show that cortical cultures grown on mwMEAs can successfully be applied to investigate the effects of different (illicit) drugs on neuronal activity. Compared to investigating multiple single endpoints for neurotoxicity or neuromodulation, such as receptor activation or calcium channel function, mwMEAs can provide information on integrated aspects of drug-induced neurotoxicity more rapidly. Therefore, this approach could contribute to a faster insight in possible health risks and shorten the regulation process.

Abbreviations: 4-FA, 4-fluoroamphetamine; 5-HT, serotonin; ACh, acetylcholine; Amph, amphetamine; DA, dopamine; DAPI, 4′,6-diamidino-2-phenylindole; DIV, days *in vitro*; FBS, fetal bovine serum; GABA, γ-aminobutyric acid; GABA-R, GABA receptor; GFAP, glial fibrillary acidic protein; IC_{50}, concentration that inhibits activity by 50%; mCPP, 1-(3-chlorophenyl)piperazine; MDMA, 3,4-Methylenedioxy-*N*-methylamphetamine; mwMEA, multi-well microelectrode array; MSR, mean spike rate; MXE, methoxetamine; NPS, new psychoactive substances; NR, neutral red; TH, tyrosine hydroxylase; VGAT, vesicular GABA transporter; VGluT1, vesicular glutamate transporter 1.

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1. Introduction

Worldwide, around 5% of the adult population used an illicit drug within the last year (last-year prevalence). This number has been fairly stable over the last years. However, the prevalence of use of common illicit drugs like cocaine, amphetamine and ecstasy is slightly decreasing, possibly because new psychoactive substances (NPS) are serving as a substitute. NPS are designed to induce effects similar to common illicit drugs, but are often not regulated and therefore often referred to as 'legal highs'. Currently, around 500 different NPS are known. These NPS have the potential to pose a risk to public health (World Drug Report, 2015). European surveys amongst young adults (15–24 years) reported an increase in lifetime prevalence of NPS use from 5% in 2011 to 8% in 2014 (Flash Eurobarometer 330, 2011; Flash Eurobarometer 401, 2014). Although the number and use of NPS is increasing, limited information is available on their toxicity and associated health risks. Screening tools that determine drug potency, can aid in predicting these health risks. These tools could also contribute to early legislation of NPS before human case reports become available (Nugteren-van Lonkhuyzen et al., 2015).

Most (illicit) drugs affect the neuronal system. Therefore, determining neuroactive effects following drug exposure is of importance. For other substances, such as pharmaceuticals, the neuroactive or neurotoxic potential is often assessed using *in vivo* experiments. This is also required by international regulations (ICH, 2000; OECD, 1997). However, such *in vivo* experiments are ethically debated, expensive and time consuming. Therefore, these experiments are unsuitable for screening a large number of substances (Bal-Price et al., 2008).

For screening purposes, alternative testing strategies are required. These can start with cytotoxicity assays in neuronal cell models and continue with assays that investigate functional neuronal effects at non-cytotoxic concentrations. The investigated effects should be neuronal specific, but preferably not refer only to a particular neuronal cell type of a specific structure in the brain. Measuring neuronal activity is an example of such an effect that can be observed in all neurons. Many of the mechanisms underlying neuromodulation and neurotoxicity ultimately result in changes in neuronal activity (for review see Bal-Price et al., 2010). Therefore, in contrast to investigating several specific mode of actions, measuring neuronal activity can limit the number of *in vitro* tests necessary to demonstrate neurotoxicity of the test compound(s).

Neuronal activity can be measured *in vitro* using different techniques. Electrophysiological techniques such as patch-clamp and amperometry allow for assessing effects of drugs with millisecond resolution and high sensitivity at a single cell level. These techniques have been successfully used to measure effects of illicit drugs on *e.g.*, ionotropic $GABA_A$ receptors (Hondebrink et al., 2011, 2013, 2015) and vesicular catecholamine release (exocytosis) (Hondebrink et al., 2009). However, these techniques are labour-intensive, lack throughput, require training and often investigate effects on a single mode of action that is too specific for screening purposes.

Alternatively, the effects of substances can be assessed by extracellular field recordings using microelectrode arrays (MEAs) (Johnstone et al., 2010). MEA recordings have been used for over a decade to investigate neuronal activity and plasticity in brain slices and neuronal cultures (Steidl et al., 2006; Obien et al., 2015; Massobrio et al., 2015). Traditionally, MEAs have been used as single well systems with a relatively low-throughput. The recent development of multi-well MEAs (mwMEAs) with 12-, 48-, or 96-wells has increased the throughput considerably. The application of these mwMEAs in neurotoxicity testing is relatively new. Several studies have recently shown that primary cortical cultures grown on mwMEAs can be used to assess the effects of specific substances with high sensitivity and specificity. These include chemicals, marine neurotoxins and some neuroactive pharmaceuticals (Puia et al., 2012; McConnell et al., 2012; Valdivia et al., 2014; Nicolas et al., 2014). Primary cortical cultures mainly consist of GABAergic and glutamatergic neurons and astrocytes (Herrero et al., 1998). However, a thorough characterisation of this frequently used cortical culture with respect to the response to neurotransmitters and neurotransmitter receptor (ant)agonists is not yet available. In addition, analysis of neuronal activity is often performed by only examining the effect following a specific exposure duration, whereas a more dynamic effect assessment may yield insight in transient effects.

We therefore first investigated the effect of several neurotransmitters and neurotransmitter receptor (ant)agonists on neuronal activity. In parallel, we investigated the effect of different analysis strategies. In addition, we investigated the effects of (illicit) drugs on neuronal activity to determine the applicability of mwMEAs as a high-throughput *in vitro* screening tool for predicting drug potency.

2. Methods

2.1. Chemicals

DL-3,4-methylenedioxy-*N*-methylamphetamine (MDMA, CAS 64057-70-1, purity 98.5%), 1-(3-chlorophenyl)piperazine (mCPP, CAS 13078-15-4, purity 98.5%), DL-4-fluoroamphetamine (4-FA, CAS 459-01-8, purity 98.5%) and methoxetamine (MXE, CAS 1239908-48-5, purity 97.9%) were obtained from Lipomed (Weil am Rhein, Germany). dl-amphetamine sulphate (Amph, CAS 60-13-9, purity 99.7%) was obtained from BUFA (Uitgeest, The Netherlands) and (−)-nicotine ditartrate (Tocris) from Spruyt Hillen (IJsselstein, Netherlands). Diazepam was obtained from Fagron (Waregem, Belgium). Neurobasal-A (NBA) medium, L-glutamine (200 mM), Penicillin/streptomycin (5000 U/mL–5000 mg/mL), fetal bovine serum (FBS) and B-27 supplement (without vitamin A) were obtained from Life Technologies (Bleiswijk, The Netherlands). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Stock solutions of drugs, neurotransmitters and neurotransmitter receptor (ant)agonists were freshly prepared in FBS medium, unless otherwise specified. The pH of stock solutions was adjusted to ~pH 7.5, if necessary.

2.2. Isolation and culture of cortical neurons

Cortical neurons were isolated from the cortex of Wistar rat pups at postnatal day 0–1 as described previously (Nicolas et al., 2014) with minor modifications. Briefly, rat pups were decapitated and the cortex was isolated and placed in dissection medium (500 mL NBA medium, supplemented with 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL glutamate (3.5 mM), 5 mL penicillin/streptomycin, 50 mL FBS and pH adjusted to 7.4). Cortices were minced and triturated to a homogenous suspension and filtered through an easy strainer (100 μm, Greiner Bio One, Alphen aan den Rijn, The Netherlands). Subsequently, cells were centrifuged for 5 min at 800 rpm. The supernatant was removed and the pellet was resuspended using 1 mL of dissection medium per rat brain and diluted to a cell suspension containing 2×10^6 cells/mL. A 50 μL drop of cell suspension (1.10^5 cells/well) was placed on the electrode field in each well of the 48-wells microelectrode array plate (MEA, Axion BioSystems Inc, Atlanta, USA, M768-GL1-30Pt200). Cultures were maintained in a humidified 5% CO_2/95% air atmosphere at 37 °C for 2 h after which 450 μL dissection medium was added to each well. For cytotoxicity testing, 100 μL of a diluted cell suspension (3×10^4

cells/well) was placed in each well of the 96-wells plate (Greiner Bio-one, Solingen Germany). Before use, all plates were coated for 2 h with poly-L-lysine (50 mg/L in sterile water).

For the mwMEA plates, the day following plating (one day *in vitro* (DIV1)), 450 μL dissection medium was replaced by 450 μL glutamate medium (450 mL NBA medium, 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL glutamate (3.5 mM), 5 mL penicillin/streptomycin and 10 mL B-27, pH 7.4). At four days *in vitro* (DIV4), 450 μL glutamate medium was replaced by 450 μL FBS medium (450 mL NBA medium, 14 g sucrose, 1.25 mL l-glutamine (200 mM), 5 mL Penicillin/streptomycin and 50 mL FBS, pH 7.4). For the 96-wells plates used for cytotoxicity measurements, dissection medium was replaced by 100 μL glutamate medium at DIV1, which was replaced by 100 μL phenol-red free FBS medium at DIV4.

For immunocytochemistry, primary cortical cultures were seeded on poly-L-lysine (50 μg/mL) coated tissue culture treated, sterile 8 chamber coverslips (Ibidi GmbH, Planegg, Germany) at a density of 1×10^5 cells/chamber.

Animal experiments were performed in agreement with Dutch law, the European Community directives regulating animal research (2010/63/EU) and approved by the Ethical Committee for Animal Experiments of Utrecht University. All efforts were made to minimize the number of animals used and their suffering.

2.3. Immunocytochemistry

Cortical cultures were fixed on DIV11-14 with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. Subsequently, coverslips were quenched for paraformaldehyde, permeabilised, and incubated with blocking buffer (2% bovine serum albumin and 0.1% saponin in PBS) containing 20 mM NH_4Cl for 20 min at room temperature. Each of the subsequent wash and incubation steps was performed in blocking buffer. Next, coverslips were incubated with one or more primary antibodies: rabbit anti-β-III tubulin, final dilution 1:500 (ab18207, Abcam, Cambridge, United Kingdom), goat anti-glial fibrillary acidic protein (GFAP) final dilution 1:100 (ab53554, Abcam, Cambridge, United Kingdom), mouse anti-vesicular GABA transporter (VGAT), final dilution 1:1000 (131 001, Synaptic Systems, Göttingen, Germany), rabbit anti-vesicular glutamate transporter 1 (VGluT1), final dilution 1:500 (ab104898, Abcam, Cambridge, United Kingdom), and/or mouse anti-Tyrosine Hydroxylase (TH), final dilution 1:500 (Sigma-Aldrich, St. Louis, USA) for 24 h at 4 °C. Subsequently, coverslips were washed 3 times with blocking buffer and incubated with one or more corresponding secondary antibodies: donkey anti-rabbit Alexa 488, donkey anti-mouse Alexa 488, donkey anti-goat 694 and/or donkey anti-rabbit 594, final concentration 1:100 (Life Technologies, Bleiswijk, The Netherlands) for 30 min at room temperature in the dark. Nuclear staining was performed by incubating the coverslips with 4′,6-diamidino-2-phenylindole (DAPI; Life Technologies) at a concentration of 200 nM for 2 min at room temperature in the dark. The washing procedure was repeated and coverslips were sealed with FluorSave (Calbiochem, San Diego, California). Immunostained coverslips were visualized using a Leica SPEII Confocal microscope (Leica DMI4000 equipped with TCS SPE-II) using a 40× oil immersion objective (N.A. 1.4-0.7) and images were captured using Leica Application Suite Advanced Fluorescence software (LAS AF version 2.6.0; Leica Microsystems GmbH, Wetzlar, Germany).

2.4. Multi-well microelectrode array (mwMEA) recordings

Multi-well microelectrode array (mwMEA) plates contain 48-wells per plate, with each well containing 16 individual embedded nanotextured gold microelectrodes, yielding a total of 768 channels (Axion Biosystems Inc.). Recordings were made as previously described (Nicolas et al., 2014; Valdivia et al., 2014). Briefly, experiments were performed at 37 °C at DIV9-11 in glutamate-free, FBS culture medium. A 48-wells mwMEA plate was placed into the Maestro 768-channel amplifier with integrated heating system, temperature controller and data acquisition interface (Axion BioSystems Inc, Atlanta, USA). After a 5 min stabilization period, a 30 min baseline recording of spontaneous activity was started. Wells with at least one visibly active electrode after equilibration were included for experiments. After the baseline recording, a 40 min exposure recording was started to determine the effects of different substances. During the first 5 min of the exposure recording, all active wells were exposed individually to test compounds by manually pipetting 5 μL of stock solution containing the test substance to each active well. The following substances and concentrations were tested: FBS medium, water (0.1%), acetylcholine, dopamine, GABA, glutamate, nicotine, serotonin, MDMA, Amph, mCPP, 4-FA or MXE (final concentrations of 0.1–1000 μM). The tested concentrations for each substance were based on range finding experiments starting with 1, 10, 100 and 1000 μM.

In a separate set of experiments, effects of the $GABA_A$-receptor antagonist bicuculline (10 μM), the $GABA_A$-receptor agonist diazepam (1 μM), the AMPA/kainate-receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), and the *N*-methyl-D-aspartate (NMDA)-receptor antagonist MK-801 (dizocilpine; 1 μM) were tested by adding 55 μL of stock solution containing the test substance (in DMSO, final concentration 0.1%) to each well.

All conditions (*e.g.*, 1 μM GABA) were tested on neuronal cultures originating from at least 2 different isolations, in at least 2 plates (average 6 plates) and at least 9 wells (average 19 wells). The number of wells represents the number of replicates per condition (see Supplemental Table 1).

Data acquisition was performed using Axion's Integrated Studio (AxIS 1.7.8) and channels were sampled at 12.5 kHz. Signals were pre-amplified with a gain of 1200× (61 dB) and band-pass filtered at 0.2–5 kHz. This raw data was re-recorded and spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a post/pre spike duration of 3.6/2.4 ms and a spike threshold of 7 × SD of the internal noise level (rms) of each individual electrode. Further data analysis was performed on the re-recorded data.

2.5. Analysis mwMEA recordings

NeuroExplorer5 software (Nex Technologies, Madison, USA) and custom-made macros in Excel were used to further analyse the data generated from mwMEA recordings. Electrodes and wells fulfilling all the following criteria were included for analysis: (1) active electrodes, (2) stable electrodes, (3) active wells. Electrodes were considered active when the mean spike rate (MSR) was >0.1 spikes/s (0.1 Hz, 6 spikes/min). Electrodes were considered stable when the MSR during 30 min baseline, divided in 5 min time windows, did not exceed the mean MSR ± 2 × SD of the complete baseline recording. Wells were considered active when at least 1 active electrode was present. The minimum MSR we applied to identify active electrodes (>6 spikes/min) is comparable with earlier studies published which used a threshold of >5 spikes/min (see *e.g.*,McConnell et al., 2012; Lefew et al., 2013; Valdivia et al., 2014; Wallace et al., 2015).

The MSR during the baseline recording ($MSR_{baseline}$) and during the exposure recording ($MSR_{exposure}$) was determined per electrode. To determine the effect of substances, $MSR_{exposure}$ was expressed as a percentage change compared to $MSR_{baseline}$ for each electrode (paired comparison), resulting in a treatment ratio. Treatment ratios of individual electrodes were averaged per well

and subsequently per condition (*e.g.*, 1 μM GABA, using the well as statistical unit (*n*)). Within each condition, outlier wells were determined based on mean percentage $\pm 2 \times$ SD and excluded (average 14%) for further analysis. For each condition, 9–30 wells from at least two independent isolations were used.

Statistical analysis consisted of expressing the treatment ratio of exposed wells (percentage change between $MSR_{baseline}$ and $MSR_{exposure}$) normalized to the treatment ratio in control experiments; *i.e.*, the treatment ratio in control experiments was set to 100%. Normalized treatment ratios of *n* wells were averaged per condition and SEM values were calculated. Next, the effect between exposure and control groups was compared by using GraphPad Prism v5.03 (GraphPad Software, San Diego, California). Groups were compared using one-way ANOVA followed by a *post-hoc* Bonferroni test when applicable. Exposure effects were considered biologically relevant if the effect exceeded mean $\pm 1 \times$ SD of the control group and was statistically significant ($p < 0.05$). Data are expressed as mean ± SEM from *n* wells derived from *N* plates.

2.6. mwMEA recordings: time-dependent effects: synchronisation and time windows

During the first 5 min of the exposure recording, all active wells were exposed to test compounds. As only active wells were exposed (manually pipetting), the exact exposure time differs per well; *e.g.*, the first well is exposed at t = 1 min, while the last well is exposed later, *e.g.*, at t = 5 min (see Fig. 2A). Using the same absolute time window of 30 min for all wells to determine the MSR could introduce a bias. When analysis is performed on the time window 0–30 min, data prior to exposure is included for the wells that are exposed last (*e.g.*, at t = 5). In contrast, when analysis is performed on the time window 5–35 min, data from the first part of the exposure is excluded for wells that are exposed first (*e.g.*, at t = 1). Since this may hamper detection of transient effects on $MSR_{exposure}$, data was synchronised to the exact time of exposure for each individual well. After synchronisation, 30 min of the exposure recording was used for analysis. $MSR_{exposure}$ was determined by calculating the mean MSR during the whole 30 min exposure recording as well as during 5 min intervals to detect transient effects. Thus, the same experimental data was used to analyse the MSR in the complete time window (0–30 min), the last time window (25–30 min) and in successive 5 min windows (*e.g.*, 5–10 min).

2.7. Cell viability

Effects of MDMA, Amph, mCPP, 4-FA and MXE on the viability of rat cortical neurons were determined using the Neutral Red assay. This assay is based on the ability of the cells to take up Neutral Red (NR) and incorporate it into lysosomes. Experiments were performed at DIV9 by exposing cells in phenol-red free NBA medium (37 °C, 5% CO_2) for 30 min to MDMA, Amph, mCPP, 4-FA or MXE at the two highest concentrations used in mwMEA experiments. Phenol-red free NBA medium was replaced by 100 μL NR in each well for 30 min (37 °C, 5% CO_2). Next, NR was replaced in each well by 100 μL NR desorb solution for 30 min during which plates were placed on a shaking platform. NR was measured spectrophotometrically at 530/645 nm excitation/emission wavelength using a plate reader (Tecan Infinite M200 microplate; Tecan Trading Männedorf, Switzerland). Data were compared using a Student's *t*-test and considered biologically relevant if significant ($p < 0.05$) and $\pm 1 \times$ SD of the control group. Data are normalized to the control and expressed as mean percentage ±SEM from *n* wells and *N* plates.

3. Results

3.1. Characterisation of cortical cultures on multi-well microelectrode arrays (mwMEAs)

Because cortical cultures on mwMEAs are a relatively new and upcoming methodology we performed an extensive characterisation of this *in vitro* model prior to assessing effects of drugs on neuronal activity. Additionally, we investigated different methods for mwMEA data analysis, including synchronisation to the time of exposure and analysis of different time windows.

3.1.1. Immunocytochemistry

Immunocytochemistry was used to determine the presence of different cell types (*e.g.*, neurons, astrocytes) and specific neuronal cell types in the cortical cultures. The vast majority of cells stained positive for either βIII-tubulin or glial fibrillary acidic protein (GFAP), indicating the abundance of neurons and astrocytes, respectively (Fig. 1A). In addition, we demonstrate the presence of different neuronal cell types, including glutamatergic and GABAergic neurons, as indicated by the positive staining for the vesicular glutamate transporter (vGlut, Fig. 1B) and the vesicular GABA transporter (vGAT, Fig. 1C). In contrast, dopaminergic neurons are absent as demonstrated by the negative staining for tyrosine hydroxylase (data not shown). This indicates that our cortical cultures consist mainly of excitatory glutamatergic neurons, inhibitory GABAergic neurons, and astrocytes.

3.1.2. Spontaneous activity and functional characteristics of cortical cultures on mwMEA

After 4 days in culture, cortical cultures develop spontaneous activity, which is stable and maximal at DIV8-12 (de Groot et al., 2016). All experiments were therefore performed on DIV9-11. The average number of active wells (≥1 active electrode) within a

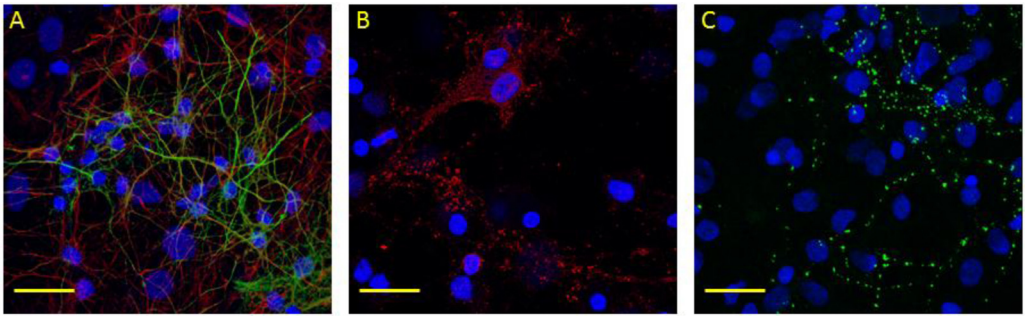

Fig. 1. Expression and distribution of (A) βIII-tubulin (green) and GFAP (red), (B) vGlut1 (red) and (C) vGAT (green) in primary rat cortical neurons as demonstrated by confocal microscopy at DIV11-14. Blue staining (DAPI) represents nuclei. Scale bar (50 μm) is indicated by the yellow line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

48-wells MEA was 28 ± 11 and the average number of active electrodes ($MSR_{baseline}$ > 0.1 spikes/s) in each well was 4 ± 3. To determine if the presence and functionality of neurotransmitter receptors in the cortical cultures can be detected using mwMEAs, we investigated its response to GABA, glutamate, nicotine, acetylcholine, serotonin and dopamine. Traditionally, MSR is determined over a 30 min exposure window or in the last 10 min of the exposure. However, our data show that some of these neurotransmitters evoke transient effects that last less than 5 min (see Section 3.1.3), precluding the use of this standard analysis. Therefore, we performed analyses at different time points within the 30 min exposure window.

3.1.3. Synchronisation and analyses of different time windows to capture transient effects

Neuronal activity may vary over time following a specific exposure, *e.g.*, due to initial activation and subsequent desensitization of neurotransmitter receptors. When analysing the complete exposure recording or by only analysing the effect at the end of the exposure, such transient effects can easily be missed. Additionally, since all wells on a mwMEA plate cannot be exposed simultaneously, the exact time at which an exposure is applied will vary for each well. We therefore synchronised the recordings to the time of exposure application for each individual well. In addition, we divided the exposure recording in windows of 5 min to analyse neuronal activity only during actual exposure (see Fig. 2 for fictional representation). This allows for detection of transient effects and for precise determination of the time at which the maximum effect occurs (see Fig. 3).

Data obtained during acetylcholine exposure confirms our hypothesis that an analysis strategy in which recordings are also analysed in short time windows (5 min) is of importance to correctly determine effects of substances on neuronal activity. Acetylcholine induces a transient increase in neuronal activity at 0.3 and 1 mM as evidenced by an increase in MSR only in specific time windows (Fig. 3). These transient effects can be highly underestimated or even undetected if not analysed in the appropriate time window. As demonstrated in Fig. 3B, exposure to 0.3 and 1 mM acetylcholine increases neuronal activity predominantly within the first 5 min of exposure (203% and 333% respectively). When the complete time window of the recording is analysed, the effect of acetylcholine is severely underestimated (98% and 140%), whereas the effect is completely absent when only the last time window (25–30 min) of the recording is analysed (93% and 83%, Fig. 3C).

To only include data following the exposure application and to precisely determine the time at which the maximum effect occurs, synchronisation to the time of exposure application is also of importance. Our data shows that the maximum effect on neuronal activity following acetylcholine exposure is increased in synchronised data compared to non-synchronised data. Synchronisation of acetylcholine data shows an increase in neuronal activity from 162% to 203% (0.3 mM) and from 308% to 333% (1 mM) (Fig. 3A and B), illustrating that effects may be underestimated using non-synchronised data.

Investigating neuronal activity in synchronised data in different time windows most accurately determines the effect of substances and clearly facilitates detecting transient effects. Therefore, all mwMEA data analyses were performed on synchronised recordings and effects were determined in consecutive 5 min time windows. For each specific exposure, the time window in which the maximum effect was observed (for data see Supplemental Table 1) was used to derive the concentration-response curves.

3.1.4. Response of cortical cultures on mwMEAs to different neurotransmitters

To determine if the presence and functionality of neurotransmitter receptors in our neuronal culture can be detected using mwMEAs, we investigated its response to several neurotransmitters compared to medium-exposed control wells. Concentration-effect curves were obtained for GABA, glutamate, nicotine, acetylcholine, serotonin and dopamine. For each concentration of each neurotransmitter, the time window at which the effect was maximal (inhibitory or stimulatory) was used for analysis (see Supplemental Table 1).

In control wells, the $MSR_{baseline}$ was 0.87 ± 0.03 spikes/s and $MSR_{exposure}$ was 0.97 ± 0.04 spikes/s. Thus, on average the number of spikes during a 30 min exposure is ~1700. The treatment ratio (change in % between $MSR_{baseline}$ and $MSR_{exposure}$) of control wells was 112 ± 1% ($n_{electrodes}$ = 1199, n_{wells} = 320, N_{plates} = 105). To quantify the effect of neurotransmitters on neuronal activity, the treatment ratio of control wells was set to 100%. Neuronal activity is affected by all tested neurotransmitters (Fig. 4). Glutamate evokes a dual effect on neuronal activity with a significant increase in MSR following exposure to 10 and 30 μM glutamate (214 ± 29% and 217 ± 32%, respectively) and a near complete inhibition of neuronal activity following exposure to ≥300 μM glutamate. High concentrations of acetylcholine (0.3 and 1 mM) increase neuronal activity to 203 ± 28% and 333 ± 52%, respectively, whereas lower concentrations do not affect activity. High concentrations of nicotine (0.3 and 1 mM) decrease neuronal activity to 29 ± 7% and 0%, respectively (Fig. 4A). GABA decreases neuronal activity already strongly at 0.3 μM and activity is completely abolished above 1 μM GABA. Neuronal activity is unaffected following serotonin exposure up to 100 μM, whereas 1 mM serotonin strongly decreases activity. Exposure to 0.1 μM dopamine increases neuronal activity, whereas higher concentrations decrease activity (Fig. 4B).

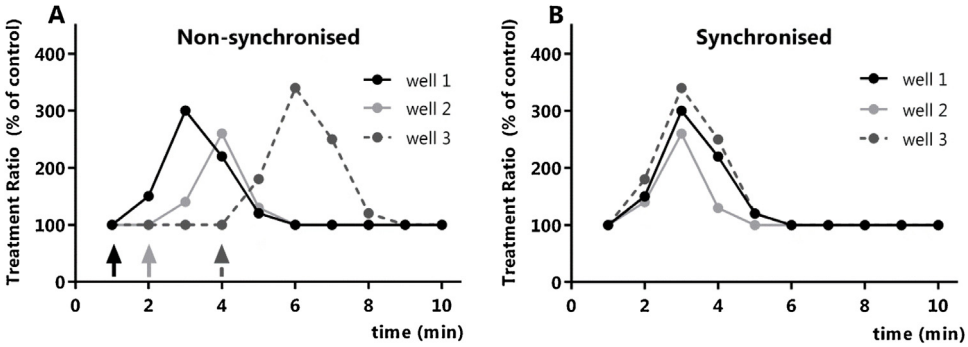

Fig. 2. Fictional representation of effects on neuronal activity over time following exposure at different time points. Arrows indicate the application of exposure. (A) Non-synchronised effects, (B) synchronised effects. Neuronal activity is expressed as the treatment ratio (change in % between $MSR_{baseline}$ and $MSR_{exposure}$) relative to control wells.

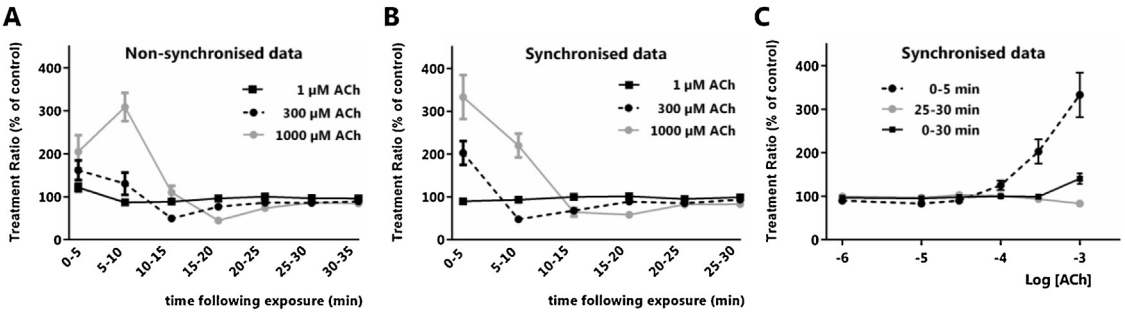

Fig. 3. Effects on neuronal activity following exposure to different concentrations of acetylcholine (ACh) within different time windows from (A) non-synchronised data, (B) synchronised to the time of exposure data, (C) synchronised data with quantification of the effect in different time windows following the onset of exposure. Results are expressed as the treatment ratio ± SEM (change in% between $MSR_{baseline}$ and $MSR_{exposure}$ relative to control wells, n_{wells} = 14–19, N_{plates} = 6–7).

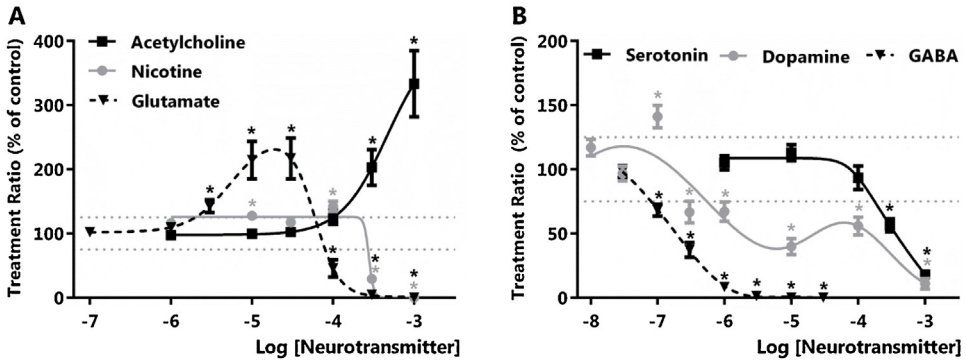

Fig. 4. Concentration-effect curves of different neurotransmitters on neuronal activity. (A) Acetylcholine (n_{wells} = 14–19, N_{plates} = 6–7), nicotine (n_{wells} = 19–26, N_{plates} = 8–11) and glutamate (n_{wells} = 21–24, N_{plates} = 7–8). (B) serotonin (n_{wells} = 17–30, N_{plates} = 4–7), dopamine (n_{wells} = 21–27, N_{plates} = 4–7) and GABA (n_{wells} = 21–25, N_{plates} = 6–7). Results are expressed as the treatment ratio ±SEM (change in % between $MSR_{baseline}$ and $MSR_{exposure}$ relative to control wells). Data were synchronised to the time of exposure and the time window in which the maximum effect was induced (Supplemental Table 1) was used for analysis. The dotted lines indicate the biological variation in control wells (±1 × SD). *$p < 0.01$.

3.1.5. Response of cortical cultures on mwMEAs to different receptor (ant)agonists

To further characterize cortical cultures on mwMEAs, we determined the effects of an antagonist and a positive allosteric modulator of $GABA_A$ receptors (bicuculline and diazepam) and two antagonists of different types of glutamate receptors (AMPA, kainate and NMDA) on neuronal activity compared to DMSO-exposed control wells. Substances were tested at a single concentration and the time window at which the effect was maximal (inhibitory or stimulatory) was used for analysis (see Supplemental Table 1).

Neuronal activity is affected by all tested substances, indicating that activation and inhibition of $GABA_A$ and glutamate receptors modulates neuronal activity (Fig. 5). Inhibition of the $GABA_A$ receptor with bicuculline strongly increases neuronal activity (254 ± 26%), while exposure to the positive allosteric modulator of the $GABA_A$ receptor diazepam strongly decreases activity (21 ± 4%). Inhibition of AMPA and kainate receptors by CNQX also strongly decreases neuronal activity (29 ± 5%), as does inhibition of NMDA receptors by MK-801 (1 ± 0.4%).

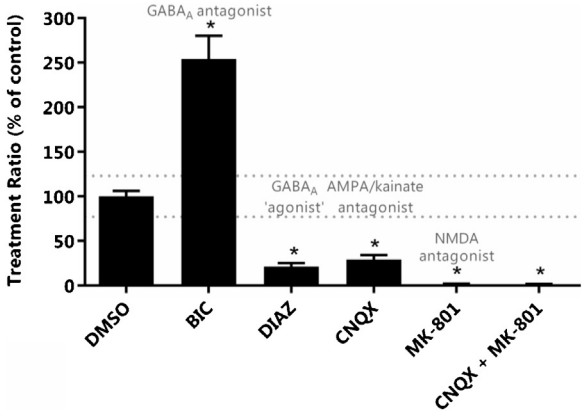

Fig. 5. Effects of different receptor (ant)agonists on neuronal activity. DMSO (n_{wells} = 15, N_{plates} = 7), BIC; bicuculline (10 µM, n_{wells} = 18, N_{plates} = 5), DIAZ; diazepam (1 µM, n_{wells} = 12, N_{plates} = 2), CNQX; 6-cyano-7-nitroquinoxaline-2,3-dione (10 µM, n_{wells} = 16, N_{plates} = 4), MK-801; dizocilpine (1 µM, n_{wells} = 19, N_{plates} = 4) and CNQX + MK-801 (10 µM and 1 µM, n_{wells} = 14, N_{plates} = 3). Results are expressed as the treatment ratio ±SEM (change in% between $MSR_{baseline}$ and $MSR_{exposure}$ relative to control wells). Data were synchronised to the time of exposure and the time window in which the maximum effect was induced (Supplemental Table 1) was used for analysis. The dotted lines indicate the biological variation in control wells (±1 × SD) *$p < 0.01$ *vs.* DMSO.

3.2. Effect of drugs

To investigate effects of acute drug exposure on neuronal activity, cortical cultures were exposed for 30 min to MDMA, Amph, mCPP, 4-FA or MXE at different concentrations. All drugs induce their effect rapidly following exposure and the effects remain stable over time (*i.e.*, non-transient). Therefore, the last 10 min of the exposure (20–30 min time window) was used for analysis.

All tested drugs inhibit neuronal activity concentration-dependently (Fig. 6). MDMA and Amph inhibit neuronal activity with comparable potency (IC_{50} 106 and 110 µM respectively) and activity is completely abolished at concentrations above 100 µM.

The NPS 4-FA is equipotent to the more commonly used drugs; a reduction in neuronal activity to 5% (±2, n = 23) was observed at 300 μM (IC_{50} 113 μM). The NPS mCPP and MXE more potently inhibit neuronal activity. mCPP decreases neuronal activity to 60% (±4, n = 21) at 30 μM, whereas higher concentrations of mCPP completely inhibit activity (IC_{50} 32 μM). Of all tested drugs, MXE most potently decreases neuronal activity; 0.3 μM already decreases activity to 59% (±4, n = 23) and 100 μM completely inhibits activity (IC_{50} 0.5 μM).

To investigate if the initial activity measured per electrode, *i.e.*, $MSR_{baseline}$, affects the degree of effect induced by the tested drugs, we also applied two different thresholds to define active electrodes. Besides the threshold of 0.1 spikes/s which is applied in all described data, we also analysed the effects of drugs using thresholds of 0.5 spikes/s and 1 spikes/s. Using these different thresholds the $MSR_{baseline}$ (spikes/s) increased from 0.86 ± 0.01 (0.1 spikes/s threshold) to 1.44 ± 0.02 and 2.04 ± 0.03 (0.5 spikes/s and 1 spikes/s threshold, respectively). Although this reduced the number of selected electrodes and thus the number of wells per condition, the drug-induced effects are comparable and thus largely independent of $MSR_{baseline}$ (Supplemental Fig. 1 and Table 2).

3.3. Cell viability

Cortical neurons were exposed to MDMA, Amph, mCPP, 4-FA or MXE at the two highest concentrations used in mwMEA experiments to determine effects on cell viability using the Neutral Red assay (membrane integrity). Only exposure to 1 mM mCPP slightly decreases the membrane integrity of cortical neurons (Fig. 7). Since neuronal activity is already completely abolished following exposure to 100 μM mCPP (Fig. 6B), the IC_{50} value for inhibition of neuronal activity by mCPP is not confounded by cytotoxicity. The cell viability data demonstrate that the observed effects on activity in mwMEA recordings during a 30 min exposure are not confounded by acute cytotoxicity.

4. Discussion

We developed an improved analysis strategy to examine effects of substances on neuronal activity of primary rat cortical cultures grown on mwMEAs. We showed that investigating the effect only at the end of the exposure can introduce false-negative results if the effect is transient. In addition, we showed that analysis should be synchronised to the time of exposure of each well to correct for the time necessary to apply different exposures within one mwMEA plate (Fig. 3). The need for synchronisation is especially high when the timing of exposure varies strongly between wells, whereas synchronisation is less indicated when multi-channel pipetting or fast robotics are used to apply exposures.

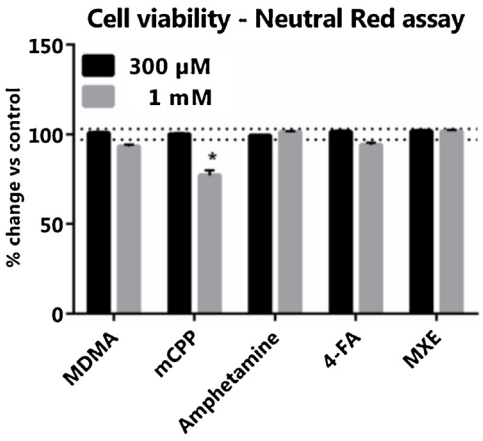

Fig. 7. Cell viability tested with the Neutral Red assay following 30 min exposure to the highest concentrations included in mwMEA experiments of MDMA (n_{wells} = 15–16, N_{plates} = 2), Amph (n_{wells} = 14–16, N_{plates} = 2), mCPP (n_{wells} = 15–16, N_{plates} = 2), 4-FA (n_{wells} = 15–16, N_{plates} = 2) or MXE (n_{wells} = 16, N_{plates} = 2). The dotted lines indicate the biological variation in control wells (±1xSD). *$p < 0.01$.

Next, we characterised this frequently used experimental model and demonstrated with immunohistochemical staining that rat cortical cultures consist of different (neuronal) cell types (Fig. 1). Using the improved analysis, we showed that neuronal activity in these cultures is affected by a range of neurotransmitters (GABA, glutamate, serotonin, dopamine, acetylcholine and nicotine) and (ant)agonists (Figs. 4 and 5). This indicates that a wide variety of receptors are present in our cortical culture, although at high concentrations neuronal activity may also be affected by non-specific effects of neurotransmitters.

The effects of most of the neurotransmitters we tested on rat cortical neuronal networks in mwMEAs were not yet described in literature. For GABA, others have also shown a decrease in neuronal activity (Gross and Rhoades, 1995; Hogberg et al., 2011). For glutamate, an increase in neuronal activity (260%) following glutamate exposure (50 μM) was reported (McConnell et al., 2012), which corresponds to our findings. In contrast to our data, others have reported that neuronal networks on mwMEAs were insensitive to nicotinic compounds such as nicotine and imidacloprid (McConnell et al., 2012; Mack et al., 2014; Valdivia et al., 2014). This lack of effect could be due to testing only a single (not effective) concentration rather than a full concentration-response curve, or

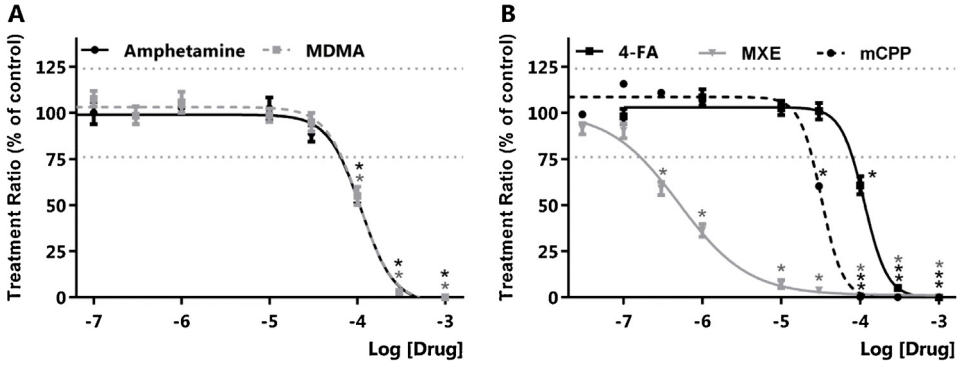

Fig. 6. Concentration-effect curves of different (illicit) drugs, including NPS. (A) MDMA (n_{wells} = 13-25, N_{plates} = 5–9) and amphetamine (n_{wells} = 13–20, N_{plates} = 5–8). (B) mCPP (n_{wells} = 11–21, N_{plates} = 5–9), 4-FA (n_{wells} = 13–23, N_{plates} = 2–5) and MXE (n_{wells} = 9–23, N_{plates} = 2–10). Results are expressed as the treatment ratio ±SEM (change in % between $MSR_{baseline}$ and $MSR_{exposure}$ relative to control wells). Data were synchronised to the time of exposure and the time window in which the maximum effect was induced was used for analysis. The dotted lines indicate the biological variation in control wells (±1 × SD). *$p < 0.01$.

because the (potentially transient) effect was only determined at the end of the exposure (Fig. 3B). For example, we showed that acetylcholine and nicotine did not affect neuronal activity below 100 μM and that the (transient) effect of acetylcholine was not detected when analysing the effect only at the end of the exposure. Although the standard approach with a single concentration and effect assessment only at the end of the exposure increases throughput, our data indicate the relevance of testing multiple concentrations and time windows to avoid false-negative results.

Notably, nicotine and acetylcholine induced opposite effects on neuronal activity. These substances can affect receptors of two subclasses (metabotropic muscarinic receptors and ionotropic nicotinic receptors) with different affinities. Consequently, nicotine and acetylcholine may evoke differential effects, in particular because ionotropic nicotinic receptors can be prone to rapid desensitization.

In addition to a thorough characterisation of this frequently used experimental model, we have investigated its applicability for examining the effects of (illicit) drugs. All tested drugs decreased neuronal activity (IC_{50} ~0.5–100 μM, Fig. 6), which was not due to cytotoxicity (Fig. 7). MDMA, amphetamine and 4-FA all had IC_{50} values around 100 μM. mCPP and methoxetamine inhibited neuronal activity more strongly with IC_{50} values of 32 and 0.5 μM respectively. The experimental model was thus able to detect effects of different types of drugs: stimulants (amphetamine, 4-FA), entactogens (MDMA), piperazines (mCPP) and dissociatives (MXE). It also showed the ability to differentiate between different drugs as is demonstrated by the varying IC_{50} values ranging from 0.5 to 113 μM. Based on the five (illicit) drugs tested so far, neuronal activity appears less affected by stimulants and entactogenic substances (IC_{50} 106–113 μM) compared to piperazines and dissociatives.

To determine if the effects detected with this method occur at relevant concentrations, we have compared IC_{50} values obtained in our experiments to blood concentrations reported in humans. When possible, we extrapolated human blood concentrations to human brain concentrations. The commonly used drugs MDMA and amphetamine decreased activity at concentrations (IC_{50} ~100 μM) that can be expected in human brain following recreational use (Hondebrink et al., 2012). Although 4-FA was equipotent in inhibiting neuronal activity as common drugs, its IC_{50} (113 μM) is well above human blood levels which were reported to be below 1 μM in driving under the influence of drugs cases (for review see Nugteren-van Lonkhuyzen et al., 2015). Investigating neuronal activity to predict the effects of 4-FA could therefore underestimate the psychoactive effects in humans. However, many drugs are known to accumulate in the brain, which results in higher brain levels. Possibly, this also occurs following 4-FA exposure, resulting in an overlap between detected effects (IC_{50}) and brain concentrations. Unfortunately, brain levels are unknown for most NPS, including 4-FA. Of the drugs more potently inhibiting neuronal activity, mCPP reduces neuronal activity (IC_{50} 32 μM) at levels that could be present in human brain. For example, recreational use of mCPP results in low micromolar blood levels in humans (Kahn et al., 1990; Klaassen et al., 1998) and a circa 30 times higher concentration could be present in the brain, as was shown in animal studies (Smith and Suckow, 1985; Miller and DeVane, 1986; DeVane et al., 1999). MXE was the most potent drug we tested. Its IC_{50} value (0.5 μM) overlaps with human serum concentrations of MXE (0.1–2 μM) following recreational use (Shields et al., 2012; Wood et al., 2011; Abe et al., 2013; Imbert et al., 2014; Łukasik-Głebocka et al., 2013). Thus, for 4 out of 5 tested drugs, neuronal activity was decreased at concentrations highly relevant for human exposure. Only 1 out of 5 tested drugs (4-FA) decreased activity at concentrations that are likely to be high for recreational human exposure. However, data on possible accumulation in the brain is lacking, hampering the comparison.

To determine neuromodulatory effects of drugs, a single mechanism of action is often investigated, in contrast to examining an integrated neuronal endpoint like neuronal activity. Examples of these single mechanisms of action include determining the inhibition of neurotransmitter reuptake transporters, activation or inhibition of neurotransmitter receptors (Simmler et al., 2014; Rickli et al., 2015) or inhibition of voltage-gated calcium channels (Hondebrink et al., 2012). When the effect concentration on reuptake transporters is compared to the effect concentration on neuronal activity, concentrations overlap for some substances (mCPP and MXE, Simmler et al., 2014; Roth et al., 2013). For other substances, inhibition of transporters occurs at a lower concentration, such as following exposure to MDMA, amphetamine and 4-FA (Rickli et al., 2015). Comparing an integrated neuronal endpoint to single mechanisms of action could provide information on the main mechanism of action. If transporter inhibition occurs at a lower concentration, this could indicate that transporter inhibition is an important mechanism of action. When effect concentrations overlap, or when neuronal activity is inhibited at lower concentrations, this could indicate other mechanisms of action are of importance. For example, MXE potently inhibits neuronal activity, also when compared to concentrations that inhibit transporter function. However, a different known mechanism of action for MXE is inhibition of the NMDA receptor (IC_{50} ~1 μM, Roth et al., 2013). Since we have shown that inhibition of NDMA receptors strongly decreases neuronal activity (Fig. 5, MK-801), this is likely the main mechanism involved in the reduced activity we have observed following MXE exposure.

The advantage of examining effects on an integrated neuronal endpoint such as neuronal activity is that the observed effect represents the sum of the effects on all mechanisms of action, *i.e.*, multiple single endpoints are investigated in a single assay. Using such an integrated endpoint can therefore provide fast insight in the effect a test substance can induce. Although the underlying mechanism often remains unknown, this method thus allows for a quick screening of a large number of substances. Likely, application of this method could reduce the number of separate experiments necessary to collect enough data on effects of novel substances to perform an initial risk assessment.

In conclusion, mwMEAs combined with cortical neurons can be used to quickly investigate neuromodulatory effects of (illicit) drugs, including NPS. Although the exact mechanism of action underlying the changes in neuronal activity remains unclear, this knowledge could provide input for regulatory processes in the field of drugs of abuse. Since the number of NPS increases yearly, with little information available on the possible adverse effects they induce, the advantage of a fast screening method such as MEA recordings is high.

Conflicts of interest

The authors do not have any conflicts of financial interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2016.04.020.

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