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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