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Neuropharmacological characterization of the new psychoactive substance methoxetamine

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ABSTRACT

The use of new psychoactive substances (NPS) is steadily increasing. One commonly used NPS is methoxetamine (MXE), a ketamine analogue. Several adverse effects have been reported following MXE exposure, while only limited data are available on its neuropharmacological modes of action.

We investigated the effects of MXE and ketamine on several endpoints using multiple *in vitro* models. These included rat primary cortical cells, human SH-SY5Y cells, human induced pluripotent stem cell (hiPSC)-derived iCell® Neurons, DopaNeurons and astrocyte co-cultures, and human embryonic kidney (HEK293) cells. We investigated effects on several neurotransmitter receptors using single cell intracellular calcium $[Ca^{2+}]_i$ imaging, effects on neuronal activity using micro-electrode array (MEA) recordings and effects on human monoamine transporters using a fluorescence-based plate reader assay.

In rat primary cortical cells, 10 μM MXE increased the glutamate-evoked increase in $[Ca^{2+}]_i$, whereas 10 μM ketamine was without effect. MXE and ketamine did not affect voltage-gated calcium channels (VGCCs), but inhibited spontaneous neuronal activity (IC_{50} 0.5 μM and 1.2 μM respectively). In human SH-SY5Y cells, 10 μM MXE slightly inhibited the K^+- and acetylcholine-evoked increase in $[Ca^{2+}]_i$. In hiPSC-derived iCell®(Dopa)Neurons, only the ATP-evoked increase in $[Ca^{2+}]_i$ was slightly reduced. Additionally, MXE inhibited spontaneous neuronal activity (IC_{50} between 10 and 100 μM). Finally, MXE potently inhibits uptake via monoamine transporters (DAT, NET and SERT), with IC_{50} values in the low micromolar range (33, 20, 2 μM respectively).

Our combined *in vitro* data provide an urgently needed first insight into the multiple modes of action of MXE. The use of different models and different (neuronal) endpoints can be complementary in pharmacological profiling. Rapid *in vitro* screening methods as those presented here, could be of utmost importance for gaining a first mechanistic insight to aid the risk assessment of emerging NPS.

1. Introduction

New psychoactive substances (NPS) are an emerging class of chemicals on the drug market. NPS are also known as 'legal highs' and mimic the psychoactive effects of illicit drugs, but are often not legislated. Over the last years, the number, type and availability of NPS is growing. In 2009, only 24 NPS were reported for the first time in Europe, increasing up to 98 additional NPS in 2015 (EMCDDA, 2016; Europol, 2013). Surveys amongst young adults showed an increase in the use of legal highs, from 5% in 2011 to 8% in 2014. In the Netherlands, 0.5% of all analyzed drug samples contained NPS in 2007, which increased to 8% in 2013 (Hondebrink et al., 2015).

In the Netherlands, methoxetamine ((RS)-2-(ethylamino)-2-(3-methoxyphenyl)cyclohexanone, MXE) was the second most frequently detected NPS in drug samples in 2013; 1.2% of all analyzed drug samples contained MXE (Hondebrink et al., 2015). In 2014, 13% of the ketamine samples offered to the Dutch Drugs Information and Monitoring System (DIMS) contained MXE (van der Gouwe, 2014). Among Dutch partygoers (15–35 years), 3% has used MXE (Linsen et al., 2015). In other countries, MXE is also a popular

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NPS with a life-time prevalence of use around 5% in the USA and the UK. Life-time prevalence of MXE use increases among ketamine users to 28% (USA) and 13% (UK) (Lawn et al., 2014).

MXE is a ketamine analogue that was sold as a 'legal' replacement for ketamine (EMCDDA-Europol, 2014). It is categorized as a hallucinogen, a class which also includes drugs like D-lysergic acid diethylamide (LSD), phencyclidine (PCP) and dextromethorphan. MXE is a dissociative anesthetic, producing derealization, sensory deprivation and dissociation from the physical body, which are all features of a 'near-death experience' and part of the desired effects of MXE (Corazza et al., 2012). Other desired effects include euphoria, feelings of peacefulness, increased empathy and social interaction and a sense of going deeper inside the self (Zawilska, 2014). These intended effects are similar to those of ketamine, but much longer lasting and with a delay in the effects (Corazza et al., 2013, 2012). As a consequence of this delay, users more frequently take a second dose, increasing the risk for adverse effects. The estimated brain concentration of MXE during recreational use is 1–5 μM (Zwartsen et al., 2017), although levels can be higher during overdose.

Multiple deaths are reported after MXE exposure, which are often associated with hyperthermia and/or the presence of other drugs of abuse (Zanda et al., 2016; Zawilska, 2014). MXE has been implicated in at least 22 fatal and 110 non-fatal intoxications, spread over eight European countries (EMCDDA and Europol, 2014; Zanda et al., 2016). MXE-induced toxicity includes psychiatric, cognitive, neurological and cardiovascular symptoms. These include drowsiness, slurred speech, anxiety, reduced ability to focus and concentrate, tremor, impaired motor coordination, aggression, hypertension and tachycardia (for review see Zawilska, 2014) as well as depressive thoughts and suicide attempts (Corazza et al., 2013).

Despite these obvious risks, the neuropharmacology of MXE is largely unknown. *In vivo* data show that MXE use is associated with bladder and renal toxicity, although MXE is advertised as a 'bladder-friendly' alternative to ketamine (Dargan et al., 2014). In rats, MXE acts as a typical dissociative anesthetic: it produces anxiogenic and stimulant effects at low doses and sedative effects at high doses. The longer lasting effects compared to ketamine described in human case reports are confirmed in rats. Moreover, MXE accumulates in the brain, which can explain the increased psychological effects (both desired and adverse) and increased toxicity compared with ketamine (Horsley et al., 2016). The limited available *in vitro* studies indicate that MXE may resemble the pharmacology of ketamine, which main mechanism is antagonism of the glutamate N-methyl-D-aspartate (NMDA) receptor (Bergman, 1999). A high binding affinity of MXE for the NMDA receptor was indeed reported (K_i 259 nM, Roth et al., 2013). While MXE also has high binding affinity for the human serotonin transporter (hSERT, Ki 0.5 μM), ketamine did not show binding affinity for hSERT at 10 μM (Roth et al., 2013). However, data demonstrating modulation of transporter or receptor function are not yet available.

Although data on the pharmacological profile of MXE are limited, human exposure and (severe) adverse effects continue to occur. Consequently, it is of importance to gain insight in the modes of action and neuropharmacological properties of MXE. *In vitro* studies are ideally suited to provide such mechanistic insight and have a higher throughput compared with *in vivo* studies, which is a clear benefit considering that full neuropharmacological profiles are hard to obtain for the rapidly increasing numbers of NPS. Most (illicit) drugs are known to affect the function of neurotransmitter receptors and monoamine transporters (Elliot & Beveridge, 2005; Rothman and Baumann, 2003), which are therefore also endpoints of interest to investigate for MXE. Functional effects on receptors can be determined by investigating effects on the intracellular calcium concentration $[Ca^{2+}]_i$ combined with specific stimuli (e.g., acetylcholine), using single cell imaging techniques (Hondebrink et al., 2012). Effects on monoamine transporters are frequently investigated using expression models in which the transporter of interest is transfected (Verrico et al., 2007; Simmler et al., 2014; Zwartsen et al., 2017). In addition to such specific modes of action, neuronal network activity has emerged as an integrated endpoint in *in vitro* neurotoxicity testing (Johnstone et al., 2010). We have previously demonstrated in rat cortical cultures that MXE is far more potent in inhibiting neuronal network activity (IC_{50} 0.5 μM) than common drugs of abuse like amphetamine and 3,4-methylenedioxymethamphetamine (MDMA) and another NPS, 4-fluoroamphetamine (Hondebrink et al., 2016). Whether MXE is as potent in a human *in vitro* model, is currently unknown. Since *in vitro* neurotoxicity testing is shifting towards using models of human origin (Wang, 2015; Shinde et al., 2016; Schmidt et al., 2017), we selected several models of human origin, including human embryonic kidney cells (HEK293), a human neuronal cell line (SH-SY5Y), human induced pluripotent stem cell (iPSC)-derived neurons and iPSC-derived astrocytes. Since MXE is a ketamine analogue, we also investigated neuropharmacological effects of ketamine.

In summary, we investigated the effects of MXE and ketamine at pharmacologically and toxicologically relevant concentrations on the function of different neurotransmitter uptake transporters, ion channel- and neurotransmitter receptor-mediated calcium entry and spontaneous neuronal activity in different human *in vitro* models.

2. Materials and methods

2.1. Chemicals

Methoxetamine (MXE, purity >97%) was obtained from Lipomed (Weil am Rhein, Germany). Ketamine was obtained from Eurocept Pharmaceuticals. Fura-2 AM was obtained from Molecular Probes (Invitrogen, Breda, The Netherlands). Neurobasal®-A (NBA) Medium, L-glutamine, fetal bovine serum (FBS), B-27 supplement, KnockOut Serum Replacement, 50/50 DMEM/F12 medium and penicillin-streptomycin (Pen/Strep) (10,000 U/mL-10000 μg/mL) were purchased from Life Technologies (Bleiswijk, The Netherlands). iCell® Neurons Maintenance Medium (NRM-100-121-001) and iCell® Neurons Medium Supplement (NRM-100-031-001) were purchased from Cellular Dynamics International (Madison, WI, USA). All other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Stock solutions of MXE (0.1 M) and ketamine (0.1 M) were prepared in saline and stored at 4 °C for a maximum of 2 weeks. Working solutions were prepared in saline just before use. Hank's Balanced Salt Solution (1X) (HBSS) buffer solution in H_2O (cell culture grade) was prepared with addition of 20 mM HEPES.

2.2. Cell culture

All cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C.

2.2.1. Rat primary cortical cells

Rat primary cortical cells were isolated from the neonatal cortex from post-natal day (PND) 1 Wistar rat pups as described previously (de Groot et al., 2016; Hondebrink et al., 2016). Briefly, rat pups were decapitated and cortices were rapidly dissected on ice. Tissues were kept in dissection medium containing NBA medium, supplemented with 25 g/L sucrose, 450 μM L-glutamine, 30 μM glutamate, 1% Pen/Strep and 10% FBS, pH was set to 7.4. Cells were