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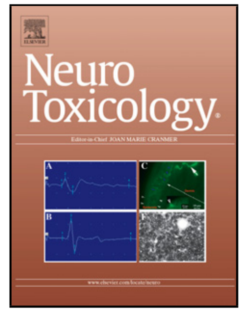

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Changes in neuronal activity in rat primary cortical cultures induced by illicit drugs and new psychoactive substances (NPS) following prolonged exposure and washout to mimic human exposure scenarios

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Highlights:

- New psychoactive substances (NPS) inhibit neuronal activity following acute exposure
- Prolonged NPS exposure did not exacerbate this concentration-dependent inhibition
- Effects of several NPS were only partly reversible following washout
- Washout of methylone increased neuronal activity at human-relevant concentrations
- Hazard characterization should include acute exposure and investigate reversibility

Abstract

The use of new psychoactive substances (NPS) is increasing despite associated health risks and limited pharmacological and toxicological knowledge. Information is available mainly for acute effects on specific targets like monoamine transporters and receptors. Recently, we have shown the ability of several NPS and illicit drugs to modulate neuronal activity during acute exposure. While these acute measurements provide valuable information regarding the potency and possible structure-activity relationships, an exposure scenario more representative of human exposure would increase insight and aid translation to the human situation.

Therefore, we investigated the effects on neuronal activity after acute (30 min) and prolonged (5 h) exposure to amphetamine-type stimulants, cathinones, hallucinogens, piperazines and cocaine using rat primary cortical cultures grown on multi-well microelectrode arrays. To investigate the reversibility of effects, activity was also measured after a washout period of 19 h.

During acute exposure, all compounds concentration-dependently decreased neuronal activity. Compared to acute exposure, prolonged exposure did not further decrease neuronal activity. Following washout, effects of 3 out of 11 drugs (methamphetamine, cocaine, and benzylpiperazine) were fully reversible, whereas effects induced by MDMA, PMMA and α-PVP were partially reversible. Neuronal activity did not recover after 19 h washout following exposure to the highest concentration of MDPV, 2C-B, 25B-NBOMe, and TFMPP. On the contrary, exposure to low concentrations of methylone, and to some extent of 2C-B, increased neuronal activity after the washout period.

Hazard characterization of emerging NPS should include at least an acute exposure to determine a potency rank order. Supplementing the (acute and prolonged) exposure scenario with a washout period allows investigation of the reversibility of effects. The possibility of a neuronal network to regain activity after drug exposure appears independent of drug class or IC_{50} values for acute and prolonged exposure. Even though neuronal activity (partly) recovers after washout following exposure to most drugs, it is perturbing that complete recovery of neuronal activity is observed only for a minority of the tested drugs.

Keywords: Designer drugs, stimulants, hallucinogens, neurotoxicology, drugs of abuse.

1. Introduction

Despite the associated health and addiction risks, the recreational use of illicit drugs remains popular. Of the adults (15-64 years) in the European Union, >25% are estimated to have used illicit drugs at least once (EMCDDA, 2018). In recent years, emerging new psychoactive substances (NPS) gained popularity as a drug of choice as alternatives for illicit drugs. Since 2009, over 800 different NPS have been reported (UNODC, 2018), which can be divided into cathinones, phenethylamines, arylcyclohexylamines, benzodiazepines, opioids, and synthetic cannabinoids. Almost 70% of these compounds were detected in the last five years and around 400 of these compounds are reported on a yearly basis (EMCDDA, 2018). The number of NPS is still increasing with around one novel NPS reported to the European monitoring center every week (EMCDDA, 2018).

Even though the emergence of NPS has been associated with severe intoxications and fatalities (UNODC, 2017), data on pharmacological, toxicological and clinical effects is often limited and/or based on a limited investigation of mechanisms of action (for review see Hondebrink et al., 2018). The best-known mechanism of action of most NPS is inhibition of monoamine reuptake transporters, which increases brain levels of dopamine, norepinephrine, and serotonin (Zwartsen et al., 2017; Rickli et al., 2015). Other targets that have been investigated include neurotransmitter receptors and ion channels, like serotonin receptors and voltage-gated calcium channels (for review see Hondebrink et al., 2018). An integrated *in vitro* system, consisting of multiple targets, that captures the complexity of the *in vivo* brain would be helpful for assessing acute neurotoxicity of the increasing number of NPS, in particular if the mechanism(s) of action are unknown *a priori*.

Recently, rat cortical cultures grown on microelectrode arrays (MEAs) have been shown to be an efficient screening tool to determine the neurotoxicity of pharmaceuticals, toxins and (environmental) chemicals (Strickland et al., 2018; Vassallo et al., 2017; Dingemans et al., 2016; Nicolas et al., 2014; Puia et al., 2012). Although the presence of monoaminergic receptors, transporters and synthesis pathways is not as pronounced in cortical cultures compared to for instance dopaminergic regions (Osredkar and Krzan, 2009), we have previously shown that these cultures are responsive to dopamine and serotonin (Hondebrink et al., 2016) and can be used to investigate the acute neurotoxicity and potency of a range of illicit drugs and NPS (Zwartsen et al., 2018; Hondebrink et al., 2016). While these acute neurotoxicity measurements provide essential data regarding the neurotoxic potency, acute (30 min) exposure is not representative of human exposure. Relevant concentrations of illicit drugs and NPS are present in human blood for several hours following exposure, as the half-life of most NPS ranges from 0.5 to 5 h in plasma (Anizan et al., 2016; Quesada et al., 2016; Rohanova and Balikova, 2009; Antia et al., 2009a; Rohanova et al., 2008; Elmore et al., 2017; Antia et al., 2009b). Notably, even such short-term exposures can induce gene and protein regulation (for review see Torres and Horowitz, 1999; Graybiel et al., 1990), and desensitization or internalization of G-protein-coupled neurotransmitter receptors (e.g. dopaminergic, adrenergic, serotonergic, glutamatergic and GABAergic receptors; Gainetdinov et al., 2004). This causes changes that can persist for hours or days (Lanahan and Worley, 1998). Additional information on the effects of drugs can thus be obtained by including prolonged exposure and assessing reversibility of effects.

In the present study, we therefore examined the effects and reversibility of effects of (illicit) drugs from several classes (amphetamine-type stimulants, cathinones, hallucinogenic phenethylamines and piperazines) following acute (30 min) and prolonged (5 h) exposure, and following washout (19 h) to more closely mimic real-life exposure scenarios.

2. Methods

2.1 Chemicals

MDMA (1-(1,3-benzodioxol-5-yl)-N-methylpropan-2-amine), PMMA (1-(4-methoxyphenyl)-N-methylpropan-2-amine), D-methamphetamine ((2S)-N-methyl-1-phenylpropan-2-amine), methylone (1-(1,3-benzodioxol-5-yl)-2-(methylamino)propan-1-one), MDPV (1-(1,3-benzodioxol-5-yl)-2-pyrrolidin-1-ylpentan-1-one), α-PVP (1-phenyl-2-pyrrolidin-1-ylpentan-1-one), 2C-B (2-(4-bromo-2,5-dimethoxyphenyl)ethanamine), 25B-NBOMe (2-(4-bromo-2,5-dimethoxyphenyl)-N-[(2-methoxyphenyl)methyl]ethanamine), BZP (1-benzylpiperazine) and TFMPP (1-[3-(trifluoromethyl)phenyl]piperazine) hydrochloride salts (purity >98.5%) were obtained from Lipomed (Weil am Rhein, Germany). Cocaine (methyl(1S,3S,4R,5R)-3-benzoloxy-8-methyl-8-azabicyclo[3.2.1]octane-4-carboxylate) (purity >98.5) was obtained from Spruyt Hillen (IJsselstein, the Netherlands). Chemical structures of the tested drugs are depicted in figure 1. Acetaminophen (purity ≥ 99%, Sigma-Aldrich (Zwijndrecht, The Netherlands)) and tetrodotoxin (TTX, Alomone Labs (Jerusalem, Israel) were used as negative and positive control, respectively. Neurobasal-A (NB-A) 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 purchased from Life Technologies (Bleiswijk, The Netherlands). All other chemicals, unless otherwise noted, were obtained from Sigma-Aldrich. Stock solutions of drugs were freshly prepared at the day of the experiment in FBS medium and acetaminophen in NB-A medium without additives. A stock solution of 10 µM TTX was made in MiliQ water and refrigerated for no longer than 2 weeks. TTX dilutions were made freshly from the stock at the day of exposure in MiliQ water.

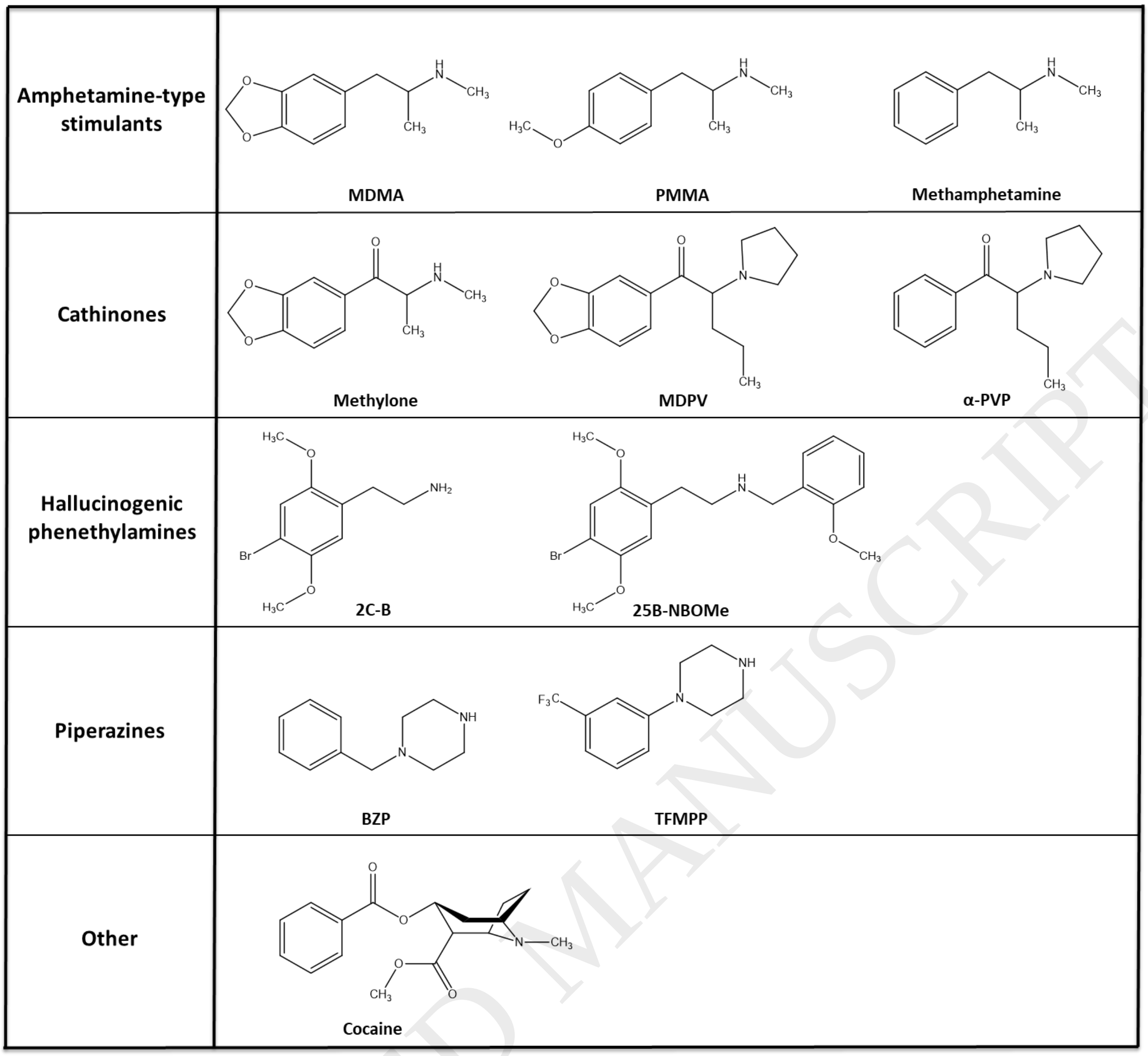

Figure. 1. Chemical structures of illicit drugs and NPS, categorized by chemical similarity.

2.2 Neuronal cultures

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.

Rat pups born of timed-pregnant Wistar rats (Envigo, Horst, the Netherlands) were sacrificed on postnatal day 0-1 to isolate cortical neurons. Cortical cultures were prepared as described previously (Zwartsen et al., 2018). Briefly, cortices were isolated, minced, triturated and filtered through a 100 µm mesh (EASYstrainer, Greiner) to get a homogenous cell suspension in dissection medium consisting of 500 mL NB-A supplemented with 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL glutamate (3.5 mM), 5 mL penicillin/streptomycin and 50 mL FBS (osmolarity of ~330 mOsm). Next, cells were centrifuged for 5 min at 800 rpm, the supernatant was removed, and the pellet was resuspended with dissection medium (1 mL per rat brain) and diluted to a cell suspension containing 2 x 10^6 cells/mL. Then, a 50 µL drop of cell suspension was added to each well (1 x 10^5 cells/well) of a 48-wells MEA plate (Axion BioSystems Inc, Atlanta, USA, M768-GL1-30Pt200) coated with PEI (0.1% PEI solution in borate buffer (24 mM sodium borate/ 50 mM boric acid in Milli-Q adjusted to pH 8.4)).

Cells were allowed to attach for 2 h at 37 ˚C, 5% CO_2/95% air atmosphere before 450 µL dissection medium was added to each well. The day after the isolation (day *in vitro* 1; DIV1), 450 µL/well of the dissection medium was replaced with 450 µL/well of glutamate medium (500 mL NB-A 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 (osmolarity of ~330 mOsm)). At DIV4, 450 µL/well glutamate medium was replaced with 450 µL/well FBS medium (glutamate-free dissection medium (osmolarity of ~330 mOsm)). Cultures were kept in FBS medium at 37 ˚C, 5% CO_2/95% air atmosphere until use at DIV9-10.

2.3 MEA recordings

As described previously (Zwartsen et al., 2018), mwMEA plates (48 wells, with 4x4 individual embedded nanotextured gold microelectrodes per well (~40-50 µm diameter; 350 µm center-to-center spacing)) were placed in a Maestro 768-channel amplifier with integrated heating system, temperature controller and data acquisition interface (Axion BioSystems Inc, Atlanta, USA) to record spontaneous neuronal activity of rat cortical cultures at DIV9-10. Prior to each recording, the plate was allowed to equilibrate for 2-5 min. Baseline spontaneous neuronal activity was recorded for 30 min at 37 ˚C. Following the baseline recording, all wells were exposed individually by manually pipetting 5 µL FBS medium without (vehicle) or with drugs, under sterile conditions. For TTX and acetaminophen, MiliQ and NB-A vehicle controls were included, respectively. Next, acute drug effects on spontaneous neuronal activity were determined during a 30 min exposure recording at 37 ˚C.

Subsequently, the plate was incubated for an additional 4 h of exposure at 37 ˚C (5% CO_2/95% air). Neuronal activity was measured again for 30 min at 37 ˚C after 4.5 h prolonged exposure. Thereafter, exposure medium was replaced with fresh FBS medium (washout) and the plate was incubated for 19 hours at 37 ˚C (5% CO_2/95% air), until the final 30 min recording, i.e., 24 hours after exposure.

The following substances and concentrations were tested: methamphetamine, methylone, BZP and TFMPP (1-1000 µM), 2C-B and α-PVP (1-300 µM), cocaine (1-100 µM), MDMA and PMMA (10-1000 µM). Due to solubility limitations of MDPV and 25B-NBOMe, wells were exposed individually by manually pipetting 55 µL FBS medium with MDPV (1-1000 µM) or without (control for MDPV), or 55 µL 10% Hanks' balanced salt solution ((HBSS)/90% NB-A) medium with 25B-NBOMe (1-30 µM) or without (control for 25B-NBOMe). For each experimental condition, primary cultures from at least two different isolations (average 3 isolations) were used and tested in at least 3 plates (average 5 plates). The number of wells represents the number of replicates per condition.

2.4 Data analysis and statistics

As described in Zwartsen et al. (2018), raw data files were re-recorded using AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) to map the spikes. Spikes were defined by > 7xSD of the internal noise level (rms) with a post/pre-spike duration of 3.6/2.4 ms of each individual electrode. Spike information was then further analyzed using NeuroExplorer® (Nex Technologies, Madison (AL), US) and custom-made macros in Excel.

To analyze effects of NPS and illicit drugs on spike, burst, network burst and synchronicity parameters (explanation of all parameters in Supplemental Table 1), only wells that contained > 4 active electrodes (≥ 0.1 spike/sec, on average 11.4 active electrodes/well) in the baseline recording were included. Bursts were defined using the Poisson surprise method (minimum of 10 surprises). Network bursts were defined using an adaptive threshold with a minimum of 40 spikes, each separated by an maximum interval set automatically on a well-by-well basis based on the mean spike rate of each well, for a minimum of 15% of the electrodes/well. Data from the last 10 min of the 30 min exposure recordings were used for analysis, since this was the most stable timeframe and no transient effects were observed in other timeframes (see Hondebrink et al., 2016 for details).

The effect on a particular parameter was calculated as follows: per well, the parameter of interest after acute exposure (or control) was expressed as a percentage of the parameter prior to exposure of the same well (the reference activity, baseline recording) to obtain a treatment ratio (paired comparison; $parameter_{exposure}/parameter_{baseline}$ as a % of control wells). The parameter after prolonged exposure and washout was also expressed as a percentage of the baseline parameter. Next, treatment ratios were grouped per parameter, condition, compound (e.g., 10 µM methylone) and exposure scenario (acute, prolonged or washout).

Wells with treatment ratios > mean ± 2xSD (of their respective condition) for the mean spike rate (MSR) were considered outliers and excluded for all parameters (on average 2.7%). Outliers in the treatment ratio for the mean burst rate (MBR; 2.7%) were excluded for all burst, network burst and synchronicity parameters. Outliers in the treatment ratio for the mean network burst rate (MNBR; 3.7%) were excluded for all network burst and synchronicity parameters. In addition, wells that were considered outliers in MSR, MBR or MNBR in the acute exposure (2.7%) were also excluded from the prolonged and the washout analysis.

Finally, the average control treatment ratios for the acute, prolonged and washout analyses were set to 100% and treatment ratios of individual exposed wells were normalized to the average treatment ratio of medium control wells of the corresponding exposure scenario. Treatment ratios of exposed wells were averaged per condition, compound and exposure scenario and used for further statistical analyses (see Zwartsen et al., 2018 for workflow on acute exposure).

As the spike, burst and network burst rates are amongst the most sensitive parameters, these parameters are presented in the manuscript, while all others are presented in the supplemental material.

GraphPad Prism software (v6, GraphPad Software, La Jolla CA, USA) was used for data analysis. Non-linear regressions were used to calculate IC_{50} values. When applicable, a one-way ANOVA followed by a *post-hoc* Dunnet's test was used to compare treatment ratios in drug-exposed wells to treatment ratios in control wells to obtain the lowest observed effect concentrations (LOECs). Effects on neuronal activity were considered relevant when the effect was statistically significant (p<0.05) and ≥ 30%. Data is shown as mean ± SEM for n_{wells} from N_{plates} from at least two different isolations.

2.5 Cytotoxicity assay

To exclude that effects of the illicit drugs and NPS on neuronal activity are due to cytotoxicity, cell viability was investigated using a Neutral Red assay (Repetto et al., 2008). Briefly, 100 µL of a cell suspension of 300.000 cells/mL was added to each well of a transparent 96-well plate (Greiner Bio-one, Solingen, Germany). The medium was changed at DIV1 and DIV4 as described for the 48-wells MEA plates, only at smaller volumes (100 µL/well). In addition, the glutamate to FBS medium change on DIV4 was done using phenol-red free NB-A medium with the above described FBS medium supplements. At DIV9-10, cells (4-6 plates from 2-4 different primary cultures) were exposed for 4.5 h to cocaine, MDMA, PMMA, methamphetamine, methylone, MDPV, α-PVP, 2C-B, 25B-NBOMe, BZP and TFMPP (final concentrations 1-1000 uM). Thereafter, the exposure medium was changed into FBS phenol-red free medium before the plates were stored at 37 ˚C, 5% CO_2/95% air atmosphere until the cell viability was tested 19.5 h later, 24 h after the start of exposure. 20 min before the NR assay, one row of cells was lysed using sodium dodecyl sulfate (SDS) to obtain background values. Medium and lysis buffer were removed from all wells after which 100 µL NR solution (Invitrogen, Breda, The Netherlands; 12 µM in phenol-red free NB-A medium w/o supplements) was added to the cells. Following 1 h incubation in the dark at 37 ˚C, the solution was removed and the cells were lysed using 100 µL NR lysis buffer (1% glacial acetic acid, 49% H_20, 50% ethanol). The plate, covered in aluminum foil, was placed on the plate shaker for approximately 30 min before fluorescence was measured using a Tecan Infinite M1000 plate reader equipped with a 10W Xenon flash light source (Tecan Group LtD; Männedorf, Switzerland). Fluorescence was measured spectrophotometrically at 530 nm excitation and 645 nm emission. Data was processed using iControl software (version 7.01).

All values were background corrected before individual control values were normalized to the average of the control wells on the corresponding plate. Next, the normalized control values of a set of experiments for a particular compound were checked for outliers (6%; average ± 2xSD). Thereafter, exposure values were normalized to the average control value (w/o outliers) on the corresponding plate. Normalized exposure values of each experimental concentration were subsequently screened for outliers (4%) based on the whole set of experiments for a particular compound. Data was processed using GraphPad Prism software and significance was determined using one-way ANOVA's followed by a *post-hoc* Dunnet's tests. Effects on cell viability were considered relevant when the effect was statistically significant ($p<0.05$) and ≥ 10%. Data is shown as mean ± SEM for n_{wells}, N_{plates} from at least two different isolations.

3. Results

3.1 Effect of illicit drug and NPS on neuronal activity

Concentration-effect curves for inhibition of neuronal activity by illicit drugs and NPS were made during acute (30 min) and prolonged (4.5 h) exposure as well as after a 19 h washout period (*i.e.* 24 h after the start of exposure). Acetaminophen exposure (10-1000 µM, negative control) had no effect on cell viability or neuronal activity (Supplemental Fig. 1A and Supplemental Fig. 2A-C). TTX exposure (1-30 nM, positive control) concentration-dependently decreased neuronal activity at non-cytotoxic concentrations (Supplemental Fig. 1B and Supplemental Fig. 2D-F). After washout, neuronal activity fully recovered following TTX exposure, highlighting the importance of additional exposure scenarios, including recovery.

The acute exposure data were derived from our previous work (Zwartsen et al., 2018) and were re-analyzed to meet the criteria as described in the methods section. All NPS and illicit drugs affected neuronal activity concentration-dependently. Mean spike rate (MSR), mean burst rate (MBR) and the mean network burst rate (MNBR) were amongst the most sensitive parameters (Supplemental Figs. 3-5) and were therefore used for detailed effect analysis. Effects on other investigated parameters are summarized as heat maps in the supplemental results (Supplemental Figs. 3-5).

3.1.1 Amphetamine-type stimulants

All amphetamine-type stimulants (ATS: MDMA, PMMA, and methamphetamine) inhibited the MSR during acute exposure, with IC_{50} values of ~100 µM (Fig. 2; Table 1). The ATS-induced decrease in MSR was paralleled by a comparable decrease in MBR and MNBR. Increasing the exposure duration did not affect the LOEC or IC_{50} value of any ATS for MSR, MBR or MNBR (Table 1). After a 19 h washout period, neuronal activity (MSR, MBR and MNBR) showed a partial recovery compared to the acute and prolonged MDMA and PMMA exposure measurements, as evidenced by the strong right-shift in concentration-effect curves. Neuronal activity fully recovered following methamphetamine exposure, and even increased after washout of 300 µM methamphetamine exposure. This increase was most pronounced in MNBR and MBR.

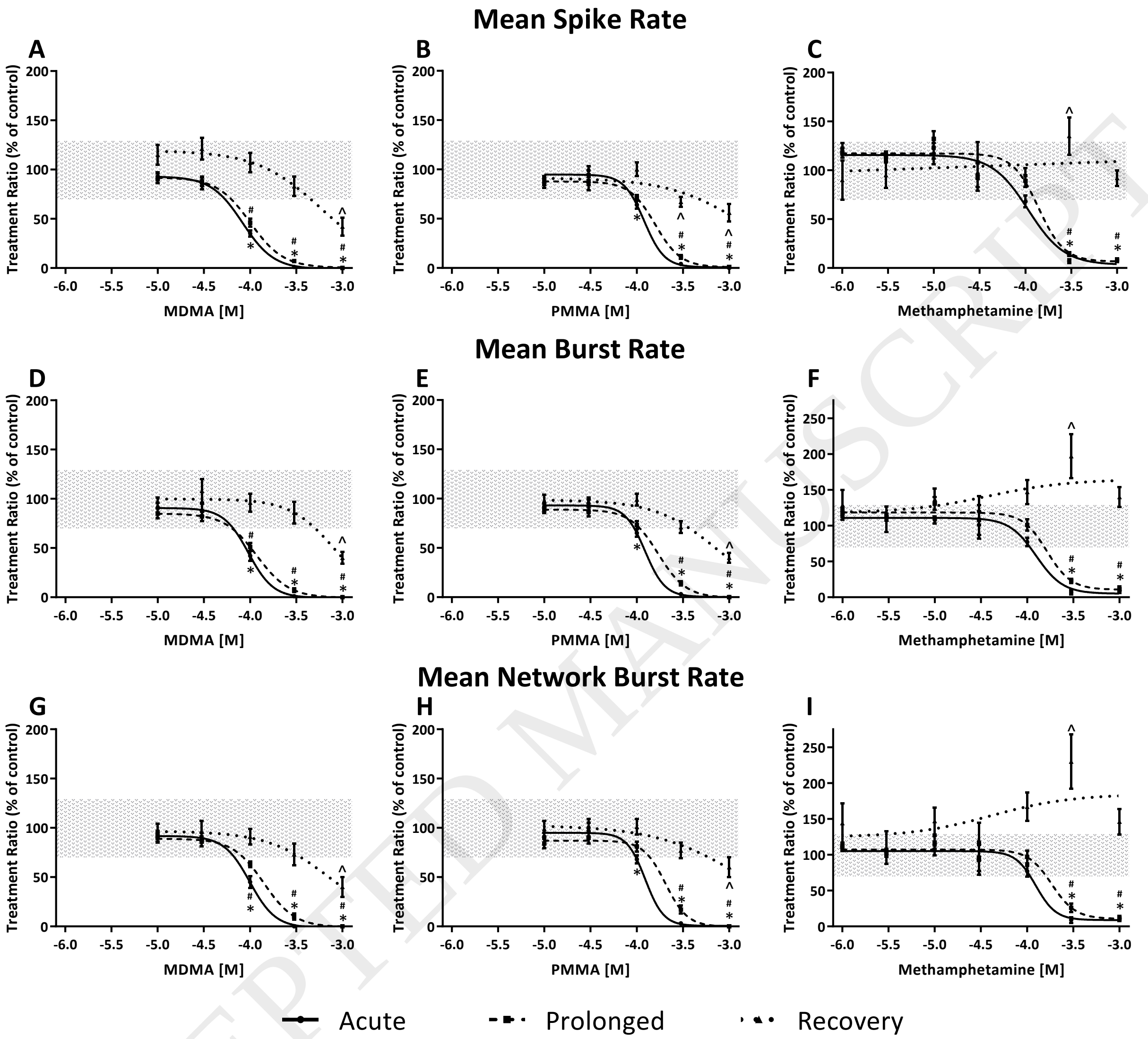

Fig. 2. Concentration-response curves of amphetamine-type stimulants for neuronal activity. The mean spike rate (MSR; A-C), mean burst rate (MBR; D-F) and mean network burst rate (MNBR; G-I) after acute exposure (solid line, 30 min), prolonged exposure (dashed line, 4.5 h) and 19 h washout (dotted line, 24 h from the start of exposure) are shown for MDMA, PMMA and methamphetamine (n_{wells}=11-25, N_{plates}=3-6). Neuronal activity is depicted as the mean treatment ratio ± SEM ($parameter_{exposure}/parameter_{baseline}$ as % of control wells). Effects ≤ 30% (*i.e.* the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects that are statistically different from control (p<0.05) are indicated with * for acute exposure, # for prolonged exposure and ^ for washout.

3.1.2 Cathinones

The cathinone methylone inhibited neuronal activity during acute exposure with IC_{50} values of 244 µM, 266 µM and 323 µM for respectively MSR, MBR and MNBR. MDPV and α-PVP were 5-10 times more potent with IC_{50} values of ~30 µM (MSR) and ~35 µM (MBR and MNBR, Fig. 3; Table 1). Prolonged exposure did not affect the potency of methylone to inhibit the MSR, MBR and MNBR compared to acute exposure. However, prolonged exposure to MDPV (MSR and MNBR) and α-PVP (MSR, MBR and MNBR) attenuated the inhibition of neuronal activity with a 2-fold maximum. Following washout of lower methylone concentrations (3-30 µM), increases in MSR, MBR and MNBR were observed. Following exposure to the highest methylone concentration, full recovery was seen, whereas no or partial recovery was seen following exposure to the highest concentration of MDPV and α-PVP, respectively. Overall, the reduction of MSR following exposure to cathinones in each exposure scenario was paralleled by a reduction in MBR and MBNR.

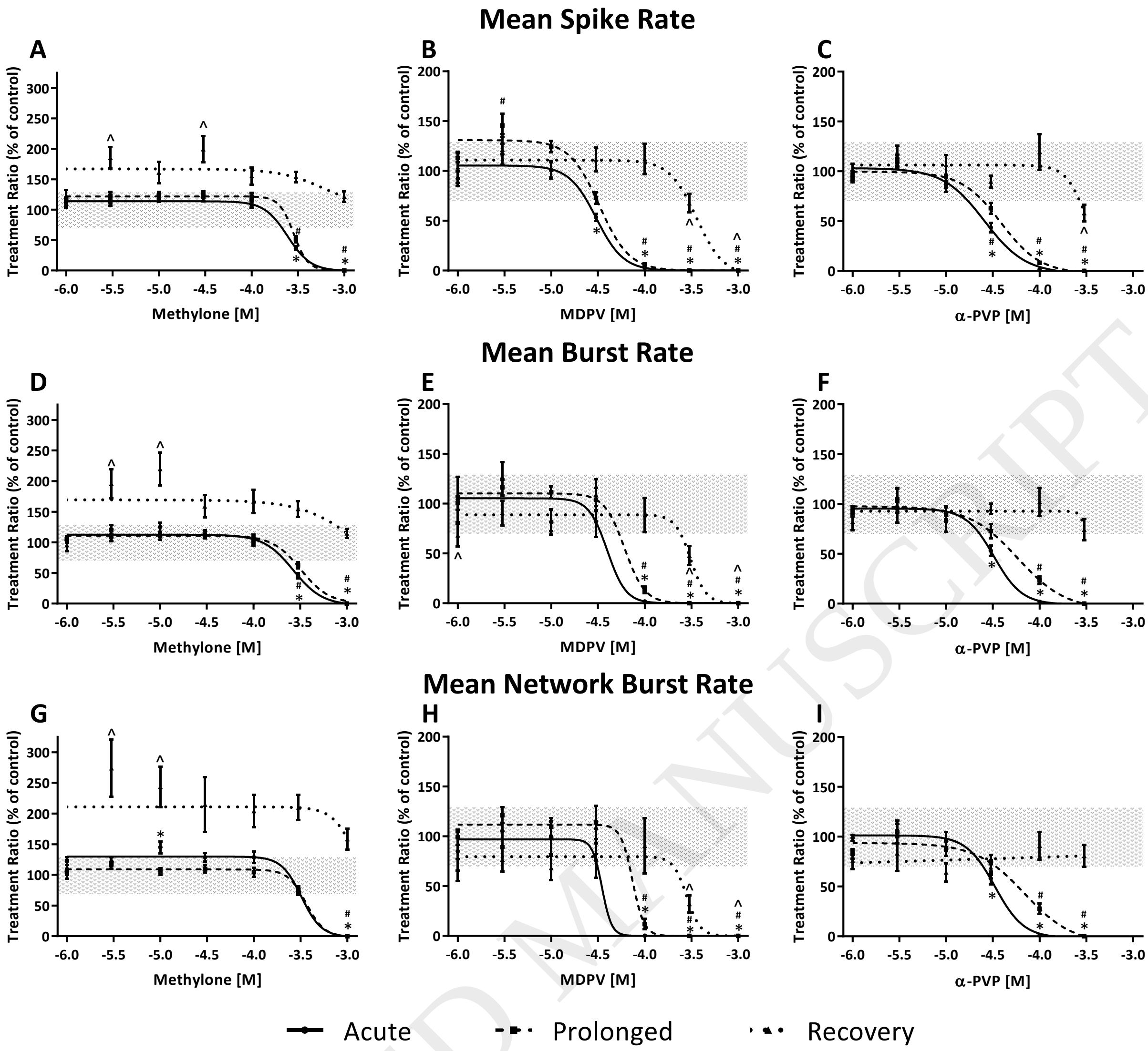

Fig. 3. Concentration-response curves of cathinones for neuronal activity. The mean spike rate (MSR; A-C), mean burst rate (MBR; D-F) and mean network burst rate (MNBR; G-I) after acute exposure (solid line, 30 min), prolonged exposure (dashed line, 4.5 h) and 19 h washout (dotted line, 24 h from the start of exposure) are shown for methylone, MDPV and α-PVP (n_{wells}=10-26, N_{plates}=3-5). Neuronal activity is depicted as the mean treatment ratio ± SEM ($parameter_{exposure}/parameter_{baseline}$ as % of control wells). Effects ≤ 30% (*i.e.* the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects that are statistically different from control (*p*<0.05) are indicated with * for acute exposure, # for prolonged exposure and ^ for washout.

3.1.3 Hallucinogenic phenethylamines

The hallucinogenic phenethylamine 2C-B inhibited neuronal activity following acute exposure with IC_{50} values ranging from 27 µM (MSR) to 39 µM (MNBR). Prolonged exposure to 2C-B slightly attenuated the inhibition of the MSR and MBR. 25B-NBOMe, which was 10-fold more potent compared to 2C-B, showed no differences between acute and prolonged exposure with respect to MSR, MBR or MNBR (Fig. 4; Table 1). After washout, a significant increase in MNBR compared to control was seen after exposure to 10 µM 2C-B, while 30 µM increased neuronal activity for both MSR and MBR. Although effects at 300 µM 2C-B were not reversible, neuronal activity recovered partially at 100 µM. Following 25B-NBOMe exposure, neuronal activity did not recover at the highest concentration (30 µM), but (partial) recovery was seen at lower concentrations.

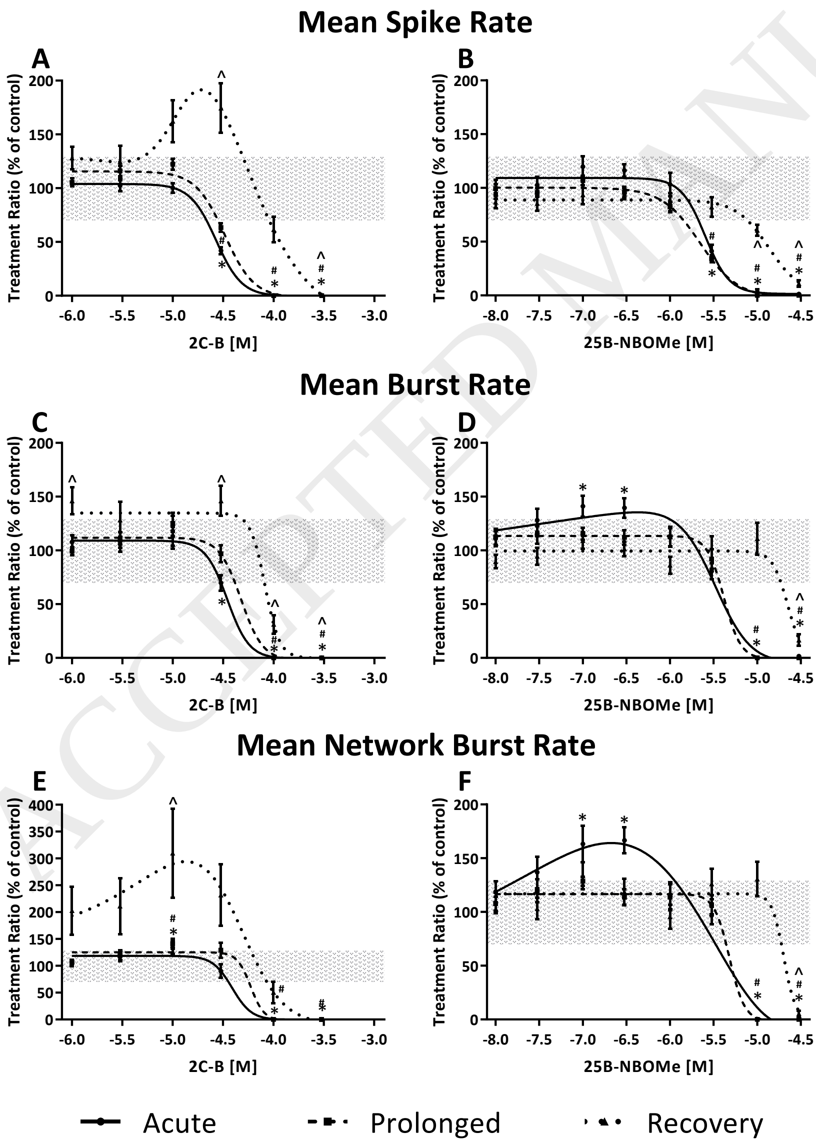

Fig. 4. Concentration-response curves of hallucinogenic phenethylamines for neuronal activity. The mean spike rate (MSR; A, B), mean burst rate (MBR; C, D) and mean network burst rate (MNBR; E, F) after acute exposure (solid line, 30 min), prolonged exposure (dashed line, 4.5 h) and 19 h washout (dotted line, 24 h from the start of exposure) are shown for 2C-B and 25B-NBOMe (n_{wells}=14-24, N_{plates}=3-4). Neuronal activity is depicted as the mean treatment ratio ± SEM ($parameter_{exposure}/parameter_{baseline}$ as % of control wells). Effects ≤ 30% (*i.e.* the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects that are statistically different from control (p<0.05) are indicated with * for acute exposure, # for prolonged exposure and ^ for washout.

3.1.4 Piperazines

TFMPP is over 10-fold more potent in inhibiting neuronal activity during acute and prolonged exposure compared to BZP (Fig. 5; Table 1). IC_{50} values for prolonged exposure were significantly increased compared to acute exposure. Following washout, effects caused by BZP exposure were fully reversible, whereas TFMPP-induced effects were only partially reversible. At 300 and 1000 µM TFMPP, neuronal activity did not recover. Notably, 1 mM TFMPP also reduced cell viability with 59% (see also section 3.2).

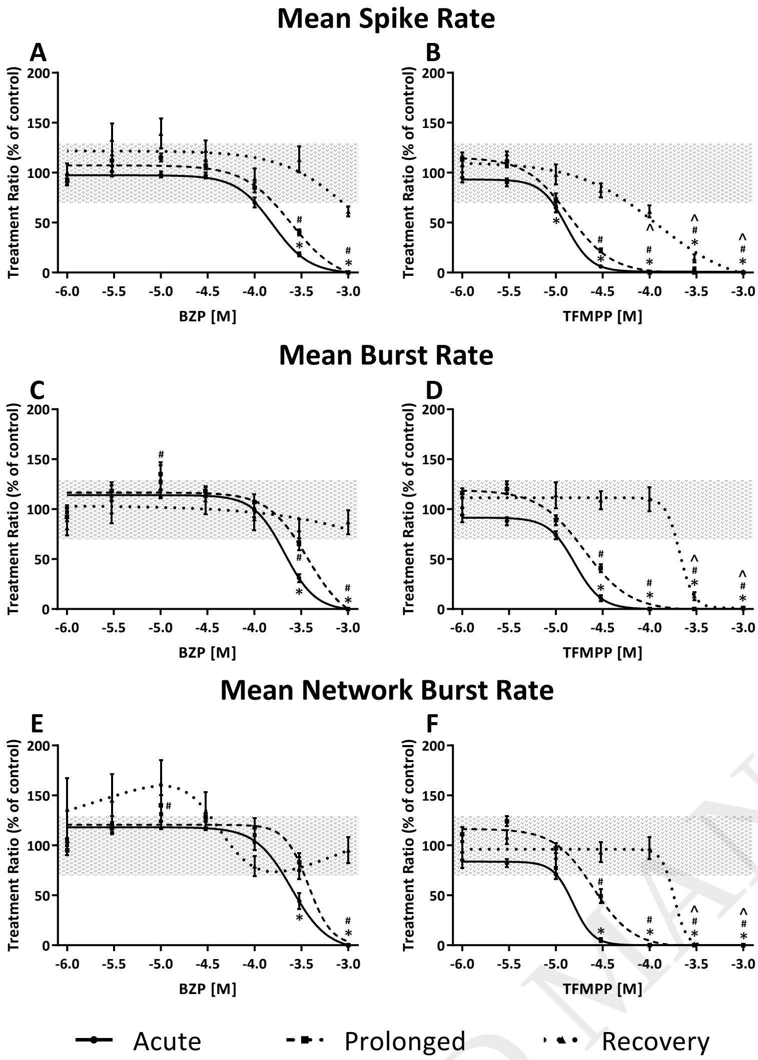

Fig. 5. Concentration-response curves of piperazines for neuronal activity. The mean spike rate (MSR; A, B), mean burst rate (MBR; C, D) and mean network burst rate (MNBR; E, F) after acute exposure (solid line, 30 min), prolonged exposure (dashed line, 4.5 h) and 19 h washout (dotted line, 24 h from the start of exposure) are shown for BZP and TFMPP (n_{wells}=14-24, N_{plates}=4-5). Neuronal activity is depicted as the mean treatment ratio ± SEM ($parameter_{exposure}/parameter_{baseline}$ as % of control wells). Effects ≤ 30% (*i.e.* the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects that are statistically different from control (p<0.05) are indicated with * for acute exposure, # for prolonged exposure and ^ for washout.

3.1.5 Cocaine

Cocaine inhibited neuronal activity with IC_{50} values of 10-15 µM during acute exposure (MSR, MBR and MNBR) and prolonged exposure (MSR) (Fig. 6; Table 1). Prolonged exposure increased the IC_{50} value to 32 and 36 µM for MBR and MNBR, respectively. LOEC values after acute and prolonged exposure are lower for MSR compared to MBR and MNBR. Neuronal activity fully recovered after the washout period for MBR and MNBR, whereas low concentrations of cocaine (1 and 3 µM) even increased the MSR after washout.

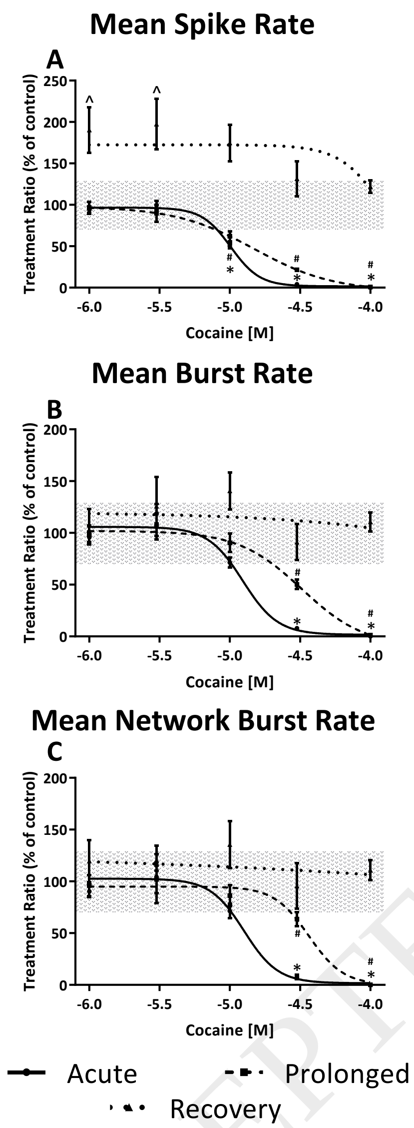

Fig. 6. Concentration-response curves of cocaine for neuronal activity. The mean spike rate (MSR; A), mean burst rate (MBR; B) and mean network burst rate (MNBR; C) after acute exposure (solid line, 30 min), prolonged exposure (dashed line, 4.5 h) and 19 h washout (dotted line, 24 h from the start of exposure) are shown for cocaine (n_{wells}=12-20, N_{plates}=4-5). Neuronal activity is depicted as the mean treatment ratio ± SEM ($parameter_{exposure}$/$parameter_{baseline}$ as % of control wells). Effects ≤ 30% (*i.e.* the variation of medium control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects that are statistically different from control (p<0.05) are indicated with * for acute exposure, # for prolonged exposure and ^ for washout.

3.2 Cell viability

Cell viability assessment (Neutral Red assay) showed that 9 out of 11 compounds did not induce cytotoxicity up to the highest concentration tested (Fig. 7). 2C-B reduced cell viability at 1 mM to 47% of control but did not reduce cell viability at 300 µM (the highest 2C-B concentration used to investigate effects on neuronal activity). In addition, TFMPP reduced cell viability at 300 µM and 1 mM to 89% and 41% of control, respectively.

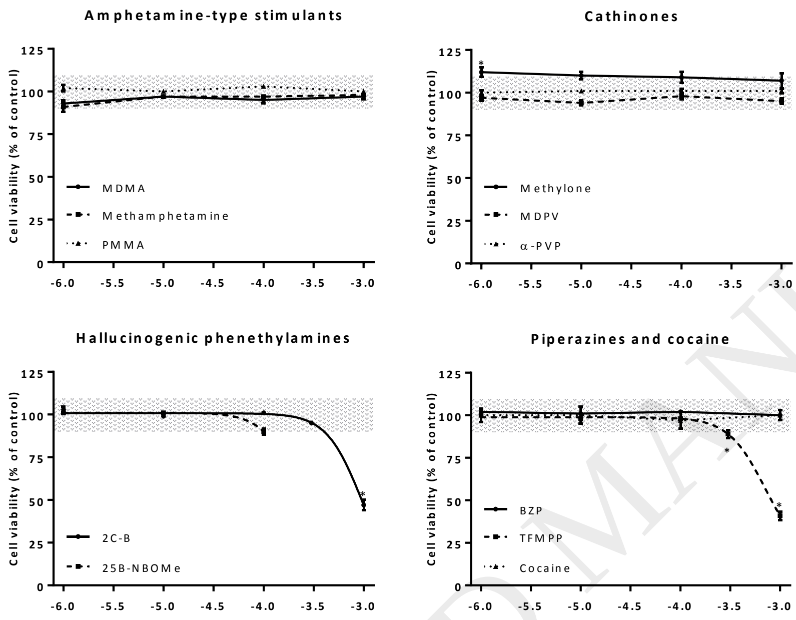

Fig. 7. Cell viability after exposure to 8 NPS and 3 illicit drugs. Neuronal cultures were exposed to 1-100 µM (25B-NBOMe) or 1-1000 µM of amphetamine-type stimulants, cathinones, hallucinogenic phenethylamines, piperazines and cocaine (n_{wells}=15-52, N_{plates}=4-10) for 4.5 h. Cell viability was assessed after 24 h using the Neutral Red assay. Effects between 90-110% are considered not to be of (toxicological) relevance, and this range is depicted by the grey area. Relevant effects that are statistically different from control (p<0.05) are indicated with *.

4. Discussion

We have previously shown that several illicit drugs and NPS concentration-dependently decrease neuronal activity after acute (30 min) exposure and that their potency to inhibit neuronal activity could be related to chemical classes or structures (Zwartsen et al., 2018). In the current research we show that prolonged exposure (4.5 h), which more closely resembles human exposure, did not further reduce neuronal activity. For some drugs, neuronal activity is even less inhibited following prolonged exposure, indicated by a small right-shift of the concentration-effect curve. This slight attenuation of inhibition of neuronal activity may be due to cellular coping mechanisms, like a change in surface expression of receptors, ion channels and/or transporters, or a lower drug availability due to e.g. sorption or metabolism. Notably, effects on neuronal spike rate (MSR) correlate well to effects on burst rate (MBR) and network burst rate (MNBR), as indicated by comparable IC_{50} values for all three parameters.

For most drugs, the inhibition of neuronal activity following (prolonged) exposure was (partly) reversible, as shown by the right-shift in concentration-effect curves of most drugs after the washout period (also see Supplemental Figs. 3-5). With the exception of TFMPP, 2C-B, and 25B-NBOMe, neuronal activity fully recovered for concentrations ≤ 100 µM. Moreover, the effects of methamphetamine, cocaine, methylone, and BZP were also fully reversible at concentrations above 100 µM. In contrast, the reduction in neuronal activity following MDMA, PMMA and α-PVP exposure above 100 µM, showed only partial (~50%) recovery and cultures exposed to high concentrations of MDPV, 2C-B, 25B-NBOMe, and TFMPP did not recover at all (also see Supplemental Fig. 3-5). For 1 mM TFMPP, this was clearly due to cytotoxicity (Fig. 7), although the lack of reversibility at 300 µM cannot be explained by only cytotoxicity. In addition, partial recovery could be due to difficulties in washing out the drugs, although this seems unlikely as the drugs have low log Kow values. Therefore, a lack of reversibility of neuronal activity following drug exposure most likely indicates persistent neurotoxic rather than cytotoxic effects.

Notably, after washout of exposure, some compounds (methamphetamine, methylone and 2C-B) even increased MSR, MBR and MNBR compared to control, albeit only at specific concentrations, while others (cocaine, BZP and MDPV) increased only one of the parameters. The ability of a neuronal network to regain activity or become overactive after exposure appears independent of drug class, and of IC_{50} values for acute and prolonged exposure. Both the ‘overcompensation’ and the loss of recovery may be due to (opposite) changes in the expression or phosphorylation of receptors and ion channels. These data thus suggest that following drug exposures, neuronal networks can behave differently and may have become resilient or more sensitive to normal stimuli or repeated (drug) exposures. Consequently, it would be interesting to include repeated exposures and longer exposure and/or recovery time as well as responsiveness to normal stimuli in future experiments.

Interestingly, 3 out of 4 drugs that showed no recovery of neuronal activity (2C-B, 25B-NBOMe and TFMPP) have a high binding affinity for 5-HT_2 receptors, while all other drugs have a low binding affinity for these receptors (Hondebrink et al., 2018; Simmler et al., 2014; Simmler et al., 2013). As the 5-HT_2 receptor is expressed in the neonatal cortex of Wistar rats (Osredkar and Krzan, 2009), it may suggest another starting point for future research.

To identify potentially harmful drugs, the effect concentrations (IC_{50} values) should be related to the concentration expected in the brain during recreational use. For 6 out of 11 drugs, the effective concentration to reduce neuronal activity is within the estimated brain concentration range during recreational use (Zwartsen et al., 2018, *i.e.* cocaine, TFMPP, MDPV, MDMA, PMMA, and methamphetamine). In addition, irreversible decreases of neuronal activity were observed within the estimated brain concentration during recreational use following exposure to TFMPP and MDMA. The concentrations of methylone that increased neuronal activity after washout are also within the estimated concentration in the brain during recreational use (Zwartsen et al., 2018). Although speculative, this could relate to the hangover following methylone exposure that users experience as one of the worst, as reported on internet drug fora.

In summary, the acute effects of drugs on neuronal activity can be investigated using short exposure scenarios (30 min) to determine a potency rank order. Increasing the exposure duration to 5 h has little additional value for potency screenings since the observed effects are in line with short exposure scenarios. However, prolonged exposure may affect the reversibility of effects and therefore could be important and requires further investigation. Investigation of the reversibility of effects is of added value, as we have shown differences in reversibility that could not be related to acute and prolonged effects, chemical structures or cytotoxicity. At concentrations relevant for human exposure, neuronal cultures exposed to TFMPP and MDMA did not recover completely, while exposure to some drugs even increased neuronal activity. Based on IC_{50} values from acute measurements, these substances were not identified as the potentially most harmful compounds. In conclusion, for (neuronal) hazard characterization of emerging NPS, exposure scenarios supplemented with experiments to investigate the reversibility of effects appears an efficient approach.

Conflict of interest

The authors declare that there are no conflicts of interest. Given his role as Editor in Chief of NeuroToxicology, Remco H.S. Westerink had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Pamela J. Lein.

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Table 1. IC_{50} values for the inhibition of the mean spike rate (MSR), mean burst rate (MBR) and mean network burst rate (MNBR) of 8 NPS and 3 illicit drugs. * depicts IC_{50} values of prolonged exposure or following washout that differ significantly from acute exposure ($p<0.05$). # depicts IC_{50} values following washout that differ significantly from prolonged exposure ($p<0.05$). IC_{50} values for MBR and MNBR significantly different from MSR at the same exposure scenario (acute, prolonged or recovery), are depicted in *italic* ($p<0.05$; > values are not taken into account). IC_{50} values for MNBR and MBR at the same exposure scenario (acute, prolonged or recovery) did not significantly differ ($p<0.05$).

Neuronal network exposure IC_{50} (µM)		Amphetamine-type stimulants			Cathinones			Hallucinogenic phenethylamines		Piperazines		Other
		MDMA	PMMA	Methamphetamine	Methylone	MDPV	a-PVP	2C-B	25B-NBOMe	BZP	TFMPP	Cocaine
MSR	Acute	83 [68-96]	120 [111-131]	107 [83-131]	244 [210-275]	30 [25-38]	26 [21-32]	27 [24-29]	2.5 [1.9-3.1]	156 [128-192]	13 [12-14]	10 [9.4-11]
	Prolonged	100 [83-123]	158* [134-183]	138 [115-173]	283 [240-313]	33* [27-40]	39* [31-51]	33* [30-36]	2.2 [1.9-2.6]	246* [186-378]	14 [12-16]	15 [9.9-35]
	Recovery	600*# [324-1122]	> 1000	> 1000	> 1000	358*# [250-558]	> 300	120*# [79-212]	14*# [10-18]	> 1000	120*# [71-674]	> 100
MBR	Acute	96 [77-114]	123 [113-136]	125 [102-156]	266 [211-366]	_40 [30-75]_	33 [28-39]	_35 [31-40]_	3.6 [3.0-4.5]	210 [159-268]	16 [14-19]	12 [11-15]
	Prolonged	118 [98-150]	170* [140-205]	163 [124-220]	322 [266-385]	_61 [45-76]_	58* [39-131]	47* [40-61]	_4.1 [3.6-5.3]_	371* [275-514]	_20* [17-23]_	_32* [21-51]_
	Recovery	805*# [503-1505]	724*# [499-1065]	> 1000	> 1000	309*# [229-403]	> 300	82*# [48-97]	22*# [16-27]	> 1000	209*# [132-259]	> 100
MNBR	Acute	99 [81-119]	124 [113-138]	124 [103-163]	323 [267-1075]	_35 [30-87]_	33 [28-42]	_39 [33-51]_	3.9 [3.1-5.6]	_248 [184-380]_	15 [13-19]	13 [11-16]
	Prolonged	_141* [117-173]_	205* [161-244]	185 [129-249]	362 [316-431]	_74* [40-87]_	69* [43-111]	59 [41-79]	_4.8 [3.9-7.0]_	374* [318-460]	_25* [31-30]_	_36* [27-51]_
	Recovery	753*# [262-1995]	> 1000	> 1000	> 1000	281*# [139-362]	> 300	> 30	20*# [14-26]	> 1000	183*# [129-249]	> 100