

# Hyperthermia exacerbates the acute effects of psychoactive substances on neuronal activity measured using microelectrode arrays (MEAs) in rat primary cortical cultures *in vitro*

Anne Zwartsen<sup>a,b</sup>, Laura Hondebrink<sup>b</sup>, Dylan W. de Lange<sup>b</sup>, Remco H.S. Westerink<sup>a,\*</sup>

<sup>a</sup> Neurotoxicology Research Group, Division Toxicology, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

<sup>b</sup> Dutch Poisons Information Center (DPIC), University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands

## ARTICLE INFO

### Keywords:

Hyperthermia  
Designer drugs  
Hazard characterization  
*In vitro* neuronal function  
Neuronal activity

## ABSTRACT

Hyperthermia is a well-known, potentially life-threatening, side effect of stimulant psychoactive substances that worsens the neurological outcome of hospitalized patients. However, current *in vitro* methods to assess the hazard of psychoactive substances do not account for hyperthermia. Therefore, this study determined the potency of five psychoactive substances (cocaine, MDMA (3,4-methylenedioxymethamphetamine), methamphetamine, 3-MMC (3-methylmethcathinone) and TFMPP (3-trifluoromethylphenylpiperazine)) to affect neuronal activity at physiological and hyperthermic conditions.

Neuronal activity of rat cortical cultures grown on microelectrode arrays (MEAs) was recorded at 37 °C before exposure. Following 30 min and 4.5 h drug exposure (1–1000 µM) at 37 °C or 41 °C, neuronal activity was measured at either 37 °C or 41 °C.

Without drug exposure, hyperthermia induced a modest decrease in neuronal activity. Following acute (30 min) exposure at 37 °C, all drugs concentration-dependently inhibited neuronal activity. Increasing the temperature to 41 °C significantly exacerbated the reduction of neuronal activity ~ 2-fold for all drugs compared to 37 °C. Prolonged (4.5 h) exposure at 41 °C decreased neuronal activity comparable to 37 °C. Neuronal activity (partly) recovered following drug exposure at both temperatures, although recovery from exposure at 41 °C was less pronounced for most drugs. None of the exposure conditions affected viability.

Since acute exposure at hyperthermic conditions exacerbates the decrease in neuronal activity induced by psychoactive substances, effects of hyperthermia should be included in future hazard assessment of illicit drugs and new psychoactive substances (NPS).

## 1. Introduction

One in twenty people between 15 and 64 years are estimated to have used at least one drug in the last year (UNODC, 2019b). After cannabis, stimulants are the most used drugs. Stimulants range from ‘classic’ illicit drugs like cocaine and 3,4-methylenedioxymethamphetamine (MDMA) to new psychoactive substances (NPS) like phenethylamines and cathinones (UNODC, 2019a).

Most illicit drugs and NPS affect the central nervous system by inhibiting the reuptake of monoamines (for review see Hondebrink et al. (2018)). The drug-induced increases in extracellular monoamines can result in intended effects (Capela et al., 2009; Glennon, 2014), but also in adverse psychiatric and cardiovascular effects (Tyrkko et al., 2016;

UNODC, 2019a).

Hyperthermia is amongst the most often reported adverse effects following exposure to classic stimulants and NPS (Greene et al., 2008; UNODC, 2019a). Presumably, this is at least partly due to drug-induced activation of cell metabolism (increasing heat production) and peripheral vasoconstriction (reducing heat dissipation) (Greene et al., 2008). As drugs are often used in warm and humid dance clubs, fatal body temperatures up to 43 °C have been reported (Greene et al., 2003).

Hyperthermia adversely affects cellular function. It exacerbates hypoxia, increases the production of reactive oxygen species (ROS) and potentiates glutamate-induced cytotoxicity, potentially leading to neuronal dysfunction, seizures, irreversible brain damage and coma (Kiyatkin, 2007; Walter and Carraretto, 2016). *In vitro*, higher

\* Corresponding author at: Neurotoxicology Research Group, Division Toxicology, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.177, NL-3508 TD Utrecht, the Netherlands.

E-mail address: [r.westerink@uu.nl](mailto:r.westerink@uu.nl) (R.H.S. Westerink).

<https://doi.org/10.1016/j.taap.2020.115015>

Received 11 December 2019; Received in revised form 15 April 2020; Accepted 17 April 2020

Available online 19 April 2020

0041-008X/ © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

temperatures stimulate the release and uptake of neurotransmitters (Nakashima and Todd, 1996; Xie et al., 2000; Volgushev et al., 2004), while the neuronal activity of rat hippocampal neurons decreased (Takeya, 2001). In addition, temperatures below the physiological range also decrease the neuronal activity in rat brain slices (Guatteo et al., 2005), indicating that both an increase and decrease in temperature can lower neuronal activity.

*In vivo* studies have shown that hyperthermia exacerbates the activation of astrocytes and the production of ROS caused by exposure to amphetamine-type stimulants (Carvalho et al., 2012; Kiyatkin, 2013). While *in vivo* studies can help determine the added neurotoxic effects of hyperthermia, the large number of NPS that have entered the market complicates (*in vivo*) screenings for hazard characterization.

Using neuronal cultures grown on microelectrode arrays (MEA), we previously determined the potency of several illicit drugs and NPS to affect neuronal activity at 37 °C (Zwartsen et al., 2018; Zwartsen et al., 2019; Zwartsen et al., 2020). MEAs non-invasively record extracellular field potentials of neuronal networks and provide different metrics describing neuronal network activity, like spike-, burst- and network burst frequency (Johnstone et al., 2010). We, therefore, used MEA recordings to determine whether hyperthermic conditions (41 °C) exacerbate alterations in neuronal activity induced by illicit drugs and NPS during acute (30 min) and prolonged (4.5 h) exposure and following washout of the drugs (19 h recovery measurement, *i.e.* 24 h after the start of the exposure).

## 2. Methods

### 2.1. Chemicals

MDMA, D-methamphetamine, 3-MMC, TFMPP and cocaine hydrochloride salts were purchased from Lipomed (Weil am Rhein, Germany) or Spruyt Hillen (IJsselstein, The Netherlands) (see Table 1 for IUPAC names, CAS numbers, purity and source). These psychoactive drugs were selected to represent the different categories (amphetamine-type stimulants, cathinones, piperazines and other) of available drugs on the drug market. Chemical structures of the tested drugs are depicted in Supplementary Fig. 1. 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 were obtained from Sigma-Aldrich. Stock solutions of drugs were freshly prepared at the day of the experiment in FBS medium.

### 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 respect the 3Rs (replacement, reduction and refinement of animals in experimental studies) by minimizing the number of animals needed and their suffering. Rat cortical cultures were used as this is currently the gold standard for neuronal activity measurements (Tukker et al., 2018).

Rat pups born of timed-pregnant Wistar rats (Envigo, Horst, The Netherlands) were sacrificed on postnatal day 0–1 and cortical cultures

were prepared as described previously in (Nicolas et al., 2014). For MEA recordings, 48-well MEA plates (Axion BioSystems Inc., Atlanta, USA) were coated with 0.1% polyethyleneimine (PEI). Next, 50  $\mu$ L cell suspension was added to each well of a 48-well MEA plate ( $1 \times 10^5$  cells/well) 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. After 2 h, 450  $\mu$ L dissection medium was added to each well. The day after cell plating (day *in vitro* 1; DIV1), 450  $\mu$ L/well dissection medium was replaced with 450  $\mu$ L/well glutamate medium (500 mL NB-A medium, 14 g sucrose, 1.25 mL L-glutamine (200 mM), 5 mL glutamate (3.5 mM); to prevent astrocyte overgrowth), 5 mL penicillin/streptomycin and 10 mL B-27 (to maintain neuronal differentiation), pH 7.4). At DIV4, 450  $\mu$ L/well glutamate medium was replaced with 450  $\mu$ L/well NB-A FBS medium (glutamate-free dissection medium).

For cytotoxicity measurements, 100  $\mu$ L rat cortical cell suspension ( $3.0 \times 10^4$  cells/well) was added to each well of a transparent 96-well plate (Greiner Bio-one, Solingen, Germany). The medium was changed at DIV1 (from dissection medium to glutamate medium) and DIV4 (from glutamate medium to NB-A FBS medium) as described for the 48-well MEA plates, using 100  $\mu$ L/well. The glutamate to FBS medium change on DIV4 was done with phenol-red free NB-A medium FBS medium to prevent interference with the fluorescence recording. For both assays, cultures were kept in NB-A FBS medium at 37 °C, 5% CO<sub>2</sub>/95% air atmosphere until use at DIV9–10.

### 2.3. MEA recordings

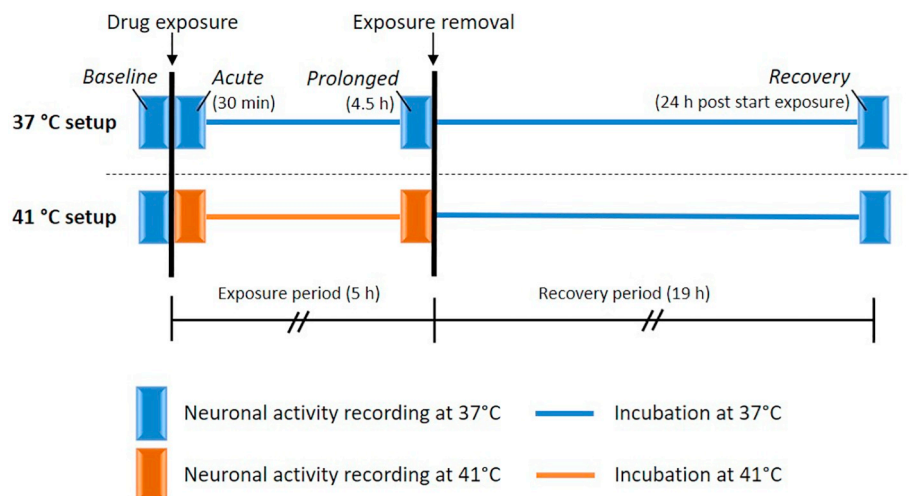
MEA recordings at 41 °C were performed as described in Zwartsen et al., 2019; (Zwartsen et al., 2020), with minor modifications. In short, neuronal activity was measured using a Maestro 768-channel amplifier (Axion BioSystems Inc., Atlanta, USA). Baseline spontaneous neuronal activity was recorded for 30 min at 37 °C. Thereafter, recording temperature was increased to 41 °C in 60 s. Next, wells were exposed in the MEA platform; each well was exposed to only one concentration of a particular drug as cumulative dosing may result in unwanted effects such as receptor desensitization. Two min following drug addition, neuronal activity was determined during a 30 min ‘acute exposure’ recording (Fig. 1). As the half-life of most illicit drugs and NPS *in vivo* ranges from 0.5 to 10 h in plasma (Jufer et al., 2000; Kalant, 2001; Antia et al., 2009; Cruickshank and Dyer, 2009; Shimshoni et al., 2015), we subsequently incubated the plate at 41 °C, 5% CO<sub>2</sub>/95% air atmosphere for 4 h, after which activity was measured during a 30 min ‘prolonged exposure’ recording (41 °C).

Next, exposure medium was replaced with fresh NB-A FBS medium and the plate was incubated for 19 h at 37 °C, until the 30 min ‘recovery’ recording (at 37 °C), which started 24 h after the start of the exposure (Fig. 1). Earlier MEA recordings, performed at 37 °C before, during and after drug exposure (Zwartsen et al. (2019); (2020)), were reanalysed for comparison.

Effects of cocaine and TFMPP were tested at 1–100  $\mu$ M, while MDMA, methamphetamine, and 3-MMC were tested at 10–1000  $\mu$ M. Concentrations were chosen based on human exposure concentrations (also see Table 2). Vehicle controls (NB-A FBS medium) were included on each plate. For each experimental condition, primary cultures from 2 to 4 different isolations were used and tested in 3–5 plates ( $N_{\text{plates}}$ ). The

**Table 1**  
Characteristics of the tested drugs.

Drug	IUPAC name	CAS number	Purity	Source
MDMA	1-(1,3-benzodioxol-5-yl)-N-methylpropan-2-amine	42542-10-09	> 98.5%	Lipomed
D-methamphetamine	(2S)-N-methyl-1-phenylpropan-2-amine	537-46-2	> 98.5%	Lipomed
3-MMC	2-(methylamino)-1-(3-methylphenyl) propan-1-one	1246816-62-5	> 98.5%	Lipomed
TFMPP	1-[3-(trifluoromethyl) phenyl]piperazine	15532-75-9	> 98.5%	Lipomed
Cocaine	methyl(1S,3S,4R,5R)-3-benzoxoy-8-methyl-8-azabicyclo[3.2.1] octane-4-carboxylate	50-36-2	> 98.5%	Spruyt Hillen



**Fig. 1.** Schematic illustration of recordings of neuronal activity at physiological (blue; 37 °C) and hyperthermic temperatures (orange; 41 °C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number of wells ( $n_{\text{wells}}$ ) represents the number of replicates per condition.

## 2.4. MEA analysis

MEA data were analysed as described in Zwartsen et al. (2019); (2020). In short, parameters of interest after acute exposure (30 min) were expressed as a percentage of the parameters prior to exposure to obtain a treatment ratio for each well (paired comparison;  $\text{parameter}_{\text{exposure}}/\text{parameter}_{\text{baseline}}$  as % of vehicle control wells). The parameters after prolonged (4.5 h) exposure and recovery (24 h after the start of the exposure) were also expressed as a percentage of the baseline parameters. Next, treatment ratios were grouped per parameter, condition, drug (e.g., weighted mean spike rate (wMSR), 10  $\mu\text{M}$  3-MMC) and exposure scenario (acute, prolonged or recovery).

Outliers ( $> \text{mean} \pm 2\text{SD}$ ) for wMSR (4.9%) were used to exclude wells on all parameters. Outliers for mean burst rate (MBR; 2.1%), and mean network burst rate (MNBR; 1.1%) were used to exclude wells on specific parameters (burst, network burst and synchronicity parameters, or network burst and synchronicity parameters, respectively). Finally, treatment ratios of exposed wells were normalized to the average treatment ratio of vehicle control wells of the corresponding parameter and exposure scenario. Thereafter, treatment ratios of exposed wells were averaged per parameter (e.g. MSR, MBR, MNBR), condition (37 °C or 41 °C), drug (MDMA, methamphetamine, 3-MMC, TFMPP or cocaine), and exposure scenario (acute, prolonged or recovery) used for further statistical analyses (see Zwartsen et al. (2019) for more details on parameter descriptions). Neuronal activity (as % of control) is expressed as  $\text{mean} \pm \text{SEM}$  of  $n_{\text{wells}}$  from  $N_{\text{plates}}$ .

## 2.5. Cytotoxicity assay

Cell viability was investigated using a Neutral Red (NR) assay as described previously (Repetto et al., 2008), with minor modifications. In short, at DIV9–10, rat cortical cells (4 plates from 2 to 3 different cultures) were exposed for 5 h at 41 °C to cocaine, MDMA, methamphetamine, 3-MMC, and TFMPP (final concentrations 1–100  $\mu\text{M}$  (cocaine and TFMPP) or 10–1000  $\mu\text{M}$  (MDMA, methamphetamine, and 3-MMC) in phenol-red free NB-A FBS medium; see methods part 2.2 *Neuronal cultures* for medium components). Thereafter, the exposure medium was changed to fresh NB-A FBS phenol-red free medium (37 °C) before the plates were stored at 37 °C, 5%  $\text{CO}_2$ /95% air atmosphere until the cell viability measurements 24 h after the start of exposure, in line with MEA experiments. At least 20 min before testing cell viability, lysis buffer (1%

glacial acetic acid, 49%  $\text{H}_2\text{O}$ , 50% ethanol) was added to non-exposed wells to obtain background values. Following the removal of medium and lysis buffer, NR solution (Invitrogen, Breda, The Netherlands; 12  $\mu\text{M}$  in phenol-red free NB-A medium w/o supplements) was added to each well. Following 1 h incubation, the NR solution was replaced with NR lysis buffer, and the plate was shaken for 20–40 min to lyse the cells. Fluorescence was measured using a Tecan Infinite M1000 plate reader equipped with a 10 W Xenon flashlight source at 530/645 nm excitation/emission wavelength.

All values were background corrected, outliers were removed in normalized control wells ( $> \text{mean} \pm 2\text{SD}$ ; 6.8%) and the exposed wells were normalized to the control values. Following the exclusion of outliers in the exposed wells (5.2%), cell viability was expressed as  $\text{mean} \pm \text{SEM}$  of  $n_{\text{wells}}$  from  $N_{\text{plates}}$ .

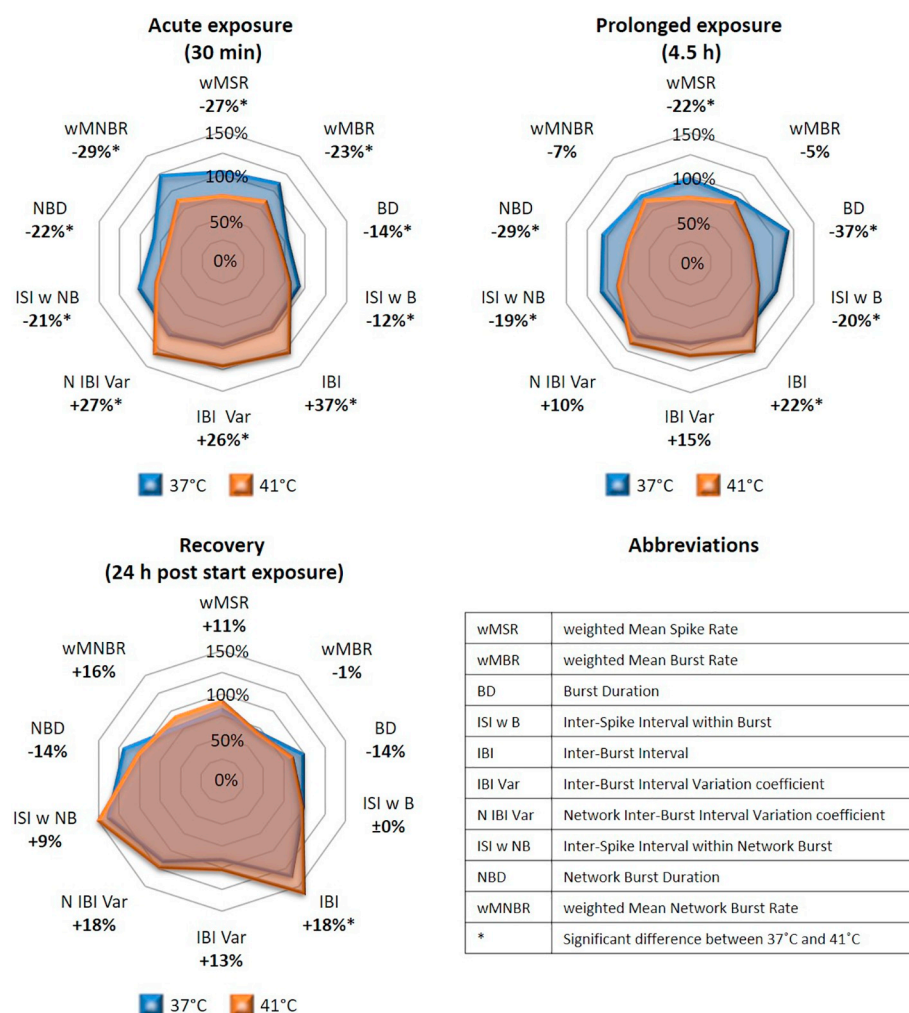
For detailed information see Zwartsen et al. (2019); (Zwartsen et al., 2020), from which the cytotoxicity data at 37 °C were re-used.

## 2.6. Statistical analysis

To make sure the baseline activity at 37 °C of cultures used to measure subsequent (drug) effects at 41 °C was not significantly different from those previously measured to determine (drug) effects at 37 °C (Zwartsen et al., 2019, 2020), an unpaired *t*-test was used to compare both data sets consisting of all relevant parameters (Graphpad Prism, version 7.04; see supplementals for all parameters).

Next, concentration-effect curves were made for MEA and cell viability data using GraphPad Prism. To calculate  $\text{IC}_{50}$  values, a four-parameter logistic curve with a variable slope was used ( $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogIC}_{50} - X) * \text{HillSlope}))}$ ). To determine significant differences between concentrations and control (at both temperatures), one-way ANOVA's followed by Dunnett's *post-hoc* tests were used (depicted by \* and # in Fig. 3). To determine significant differences between temperatures at specific concentrations (multiple) unpaired *t*-tests were used (depicted by ^ in Fig. 3). Unpaired *t*-tests were also used to determine whether differences due to temperature in  $\text{IC}_{50}$  values were significant (Table 2).

All statistical tests were performed using GraphPad Prism. As the number of wells ( $n$ ) used in this study is large, even small changes that are within the level of biological variation can reach significance. Drug-induced effects on neuronal activity and cell viability were therefore considered relevant only if the effect was statistically significant ( $p < .05$ ) and larger than the average variation of the control experiments (biological variation;  $\geq 30\%$  or  $\geq 10\%$ , for MEA and viability measurements respectively).



**Fig. 2.** Spider plots of neuronal activity at physiological (blue; 37 °C) and hyperthermic (orange; 41 °C) conditions. Neuronal activity of wells exposed to the vehicle control at 37 °C ( $n_{\text{wells}} = 73\text{--}90$ ,  $N_{\text{plates}} = 26$ ) and 41 °C ( $n_{\text{wells}} = 108\text{--}131$ ,  $N_{\text{plates}} = 22$ ) is depicted following acute (30 min) and prolonged (4.5 h) exposure, and after the recovery period (19 h, i.e. 24 h after the start of the exposure). The difference between parameters at 37 °C and 41 °C is depicted in bold. \* indicates a temperature-induced significant difference at the different exposure scenarios (acute, prolonged and recovery) ( $p < .05$ ). Neuronal activity is depicted as a % of baseline for 10 parameters. See the supplementals of Zwartsen et al. (2019) for a detailed description of all parameters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3. Results

#### 3.1. Neuronal activity at physiological and hyperthermic conditions

The baseline neuronal activity (all parameters combined) of the cultures, i.e. before assessing effects of changes in temperature, did not differ subsequently between cultures measured at 37 °C and at 41 °C ( $p = .84$ ). When temperature increased from 37 °C to 41 °C during acute (30 min) recordings, neuronal activity (weighted mean spike rate (wMSR), weighted mean burst rate (wMBR), and weighted mean network burst rate (wMNBR)) was reduced with 23–29% (Fig. 2). This is paralleled by an increase in (network) burst interval variation coefficients, indicating a more sporadic (network) burst pattern. Moreover, the (network) bursts are shorter, with spikes within the (network) bursts occurring at higher rates (Fig. 2).

With the exception of network burst duration (NBD), all parameters of neuronal activity following prolonged vehicle exposure (4.5 h) at 41 °C were comparable to acute vehicle exposure, whereas for several neuronal parameters differences were observed between acute and prolonged exposure at 37 °C (e.g. the duration and frequency of bursts and network bursts decreased). As a result, temperature-evoked differences in neuronal activity are evident following prolonged exposure.

Neuronal activity following recovery (19 h recovery, i.e. 24 h after the start of the exposure) at both temperatures was largely comparable. However, some differences were seen when comparing recovery measurements to prolonged exposure measurements. After recovery following exposure to 37 °C, the frequency of spike, burst and network burst decreased, while the inter-burst interval (IBI) and the inter-spike

interval within network burst (ISI w NB) increased. After recovery from prolonged exposure to 41 °C, comparable effects were seen, like the decrease in burst rate and the increase in inter-burst interval (IBI) and the inter-spike interval within network burst (ISI w NB). Additionally an increase in the spike frequency and the network burst duration (NBD) was seen (Fig. 2).

In conclusion, while some (biological) variation in neuronal activity is seen following exposure to 37 °C and recovery, a consistent but modest decrease in neuronal activity (% compared to baseline) is seen following acute and prolonged exposure at 41 °C. Notably, following the recovery period, neuronal networks exposed to 41 °C showed comparable activity to neuronal networks solely exposed to 37 °C.

#### 3.2. Effect of psychoactive substances on neuronal activity at hyperthermic conditions

Most parameters describing neuronal activity were concentration-dependently affected by drug exposure and differences were seen between temperatures. As the wMSR was most sensitive for exposure at higher temperatures, and all other parameters showed comparable effects (or inversely proportional effects), only effects on the wMSR are depicted in Fig. 3 (for an overview of effects on other parameters see Supplementary Fig. 2 and 3).

At 37 °C, all substances inhibited the wMSR during acute (30 min) and prolonged (4.5 h) exposure (Fig. 3, black lines).  $IC_{50}$  values for cocaine and TFMPP were  $\sim 10 \mu\text{M}$ , while  $IC_{50}$  values for the other drugs were 60–145  $\mu\text{M}$  (Table 2). Neuronal activity increased to levels above baseline following recovery (19 h post exposure, i.e. 24 h after the start

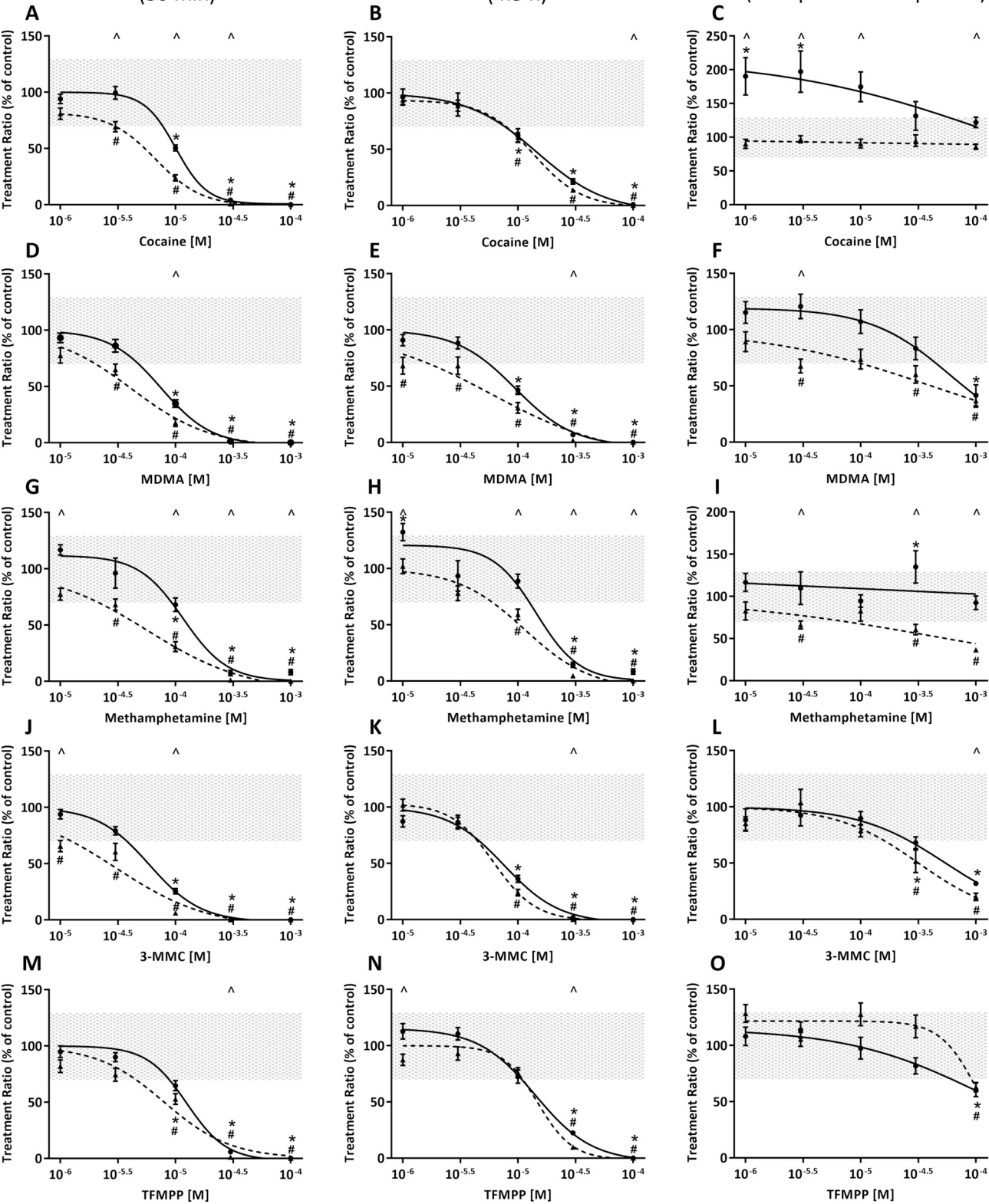
Neuronal activity (wMSR)

— 37°C      - - - 41°C

Acute exposure  
(30 min)

Prolonged exposure  
(4.5 h)

Recovery  
(24 h post start exposure)



(caption on next page)

**Fig. 3.** Concentration-effect curves of psychoactive substances for neuronal activity at physiological (37 °C; solid line) and hyperthermic (41 °C; dashed line) conditions. The wMSR after acute (left; 30 min) and prolonged (middle; 4.5 h) exposure, and recovery (right; 24 h after the start of the exposure) are shown for cocaine (A-C), MDMA (D-F), methamphetamine (G-I), 3-MMC (J-L), and TFMPP (M-O) ( $n_{\text{wells}} = 8-34$ ,  $N_{\text{plates}} = 3-7$ ). Neuronal activity is depicted as the mean treatment ratio  $\pm$  SEM. Effects  $\leq 30\%$  (i.e. the variation of vehicle control) are considered not to be of (toxicological) relevance (depicted by the grey area). Relevant effects that are statistically different from control ( $p < .05$ ) are indicated with \* for 37 °C and with # for 41 °C. ^ represents concentrations at which effects differed significantly between 37 °C and 41 °C.

of the exposure) from exposure to low cocaine concentrations at 37 °C, while higher concentrations recovered to baseline values. Neuronal networks exposed to high concentrations of MDMA, 3-MMC and TFMPP did not recover completely. Neuronal activity recovered completely following methamphetamine exposure and lower concentrations of MDMA, 3-MMC and TFMPP.

While neuronal activity is already reduced at 41 °C, the increase in temperature further exacerbated the inhibition of neuronal activity following acute exposure for all drugs (Table 2; Fig. 3, dashed lines). A close to 2-fold decrease in acute wMSR  $IC_{50}$  values was seen following exposure at 41 °C vs. 37 °C. Following prolonged exposure and recovery, no significant differences were observed between wMSR  $IC_{50}$  values for drug exposure at 37 °C or 41 °C. Neuronal networks exposed at 41 °C to cocaine, MDMA, methamphetamine and 3-MMC tend to recover less from exposure compared to exposure at 37 °C.

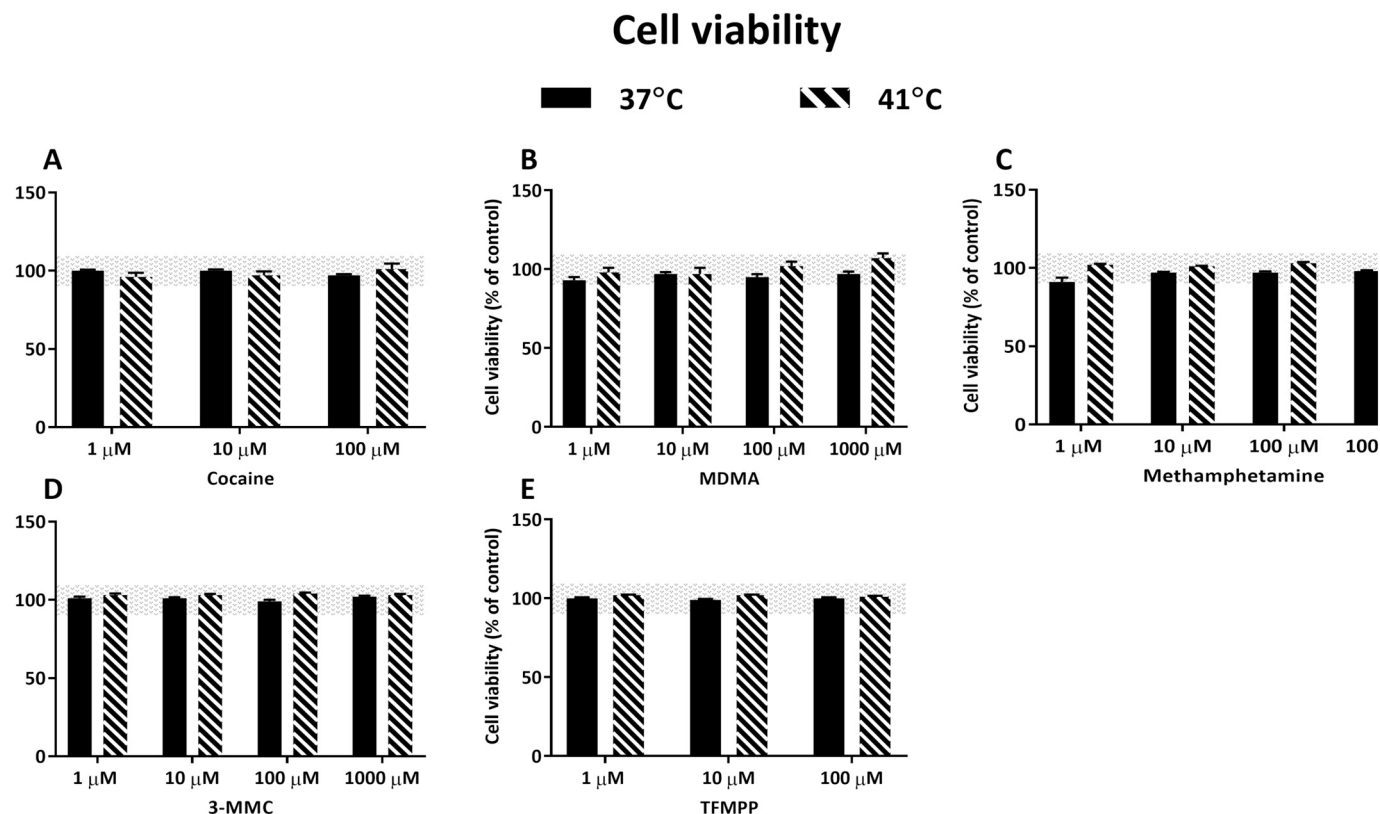
### 3.3. Lack of cytotoxic effects of psychoactive substances at hyperthermic conditions

None of the tested substances reduced cell viability at either 37 °C or 41 °C following prolonged exposure (4.5 h) to, and washout (recovery; 19 h post exposure, i.e. 24 h after the start of the exposure) from concentrations used for the assessment of effects on neuronal activity (Fig. 4).

## 4. Discussion

Neuronal activity is an efficient readout to investigate effects of xenobiotics such as pharmaceuticals, toxins, (environmental) chemicals and psychoactive drugs on neuronal function (Puia et al., 2012; Nicolas et al., 2014; Dingemans et al., 2016; Vassallo et al., 2017; Strickland et al., 2018; Zwartsen et al., 2018). To gain insight into the influence of temperature on neuronal functioning, and to relate our results to clinical situations in which drug users often suffer from hyperthermia, drug-induced effects on neuronal activity following exposure at physiological and hyperthermic conditions were compared. While hypothermia reduces neuronal activity *in vitro* (Guatteo et al., 2005), information on the effects of hyperthermia on *in vitro* neuronal function at temperatures over 38 °C is scarce. Our data show that, even without drug exposure, neuronal activity decreases (spike, burst and network burst rate) when the temperature increases from 37 °C to 41 °C (Fig. 2).

In the presence of drug exposure, the inhibition of neuronal activity (wMSR) was further exacerbated ~2-fold at 41 °C compared to drug effects at 37 °C during acute (30 min) measurements (Table 2). Exacerbation of drug effects at a higher temperature was most profound for 3-MMC and methamphetamine, while effects of TFMPP were least affected (Table 2, Fig. 3). While the exacerbation of effects at a higher temperature was largely absent following prolonged (4.5 h) drug



**Fig. 4.** Cell viability of rat cortical neurons after drug exposure at physiological (37 °C; black) and hyperthermic (41 °C; striped) conditions. Cytotoxicity was determined using a Neutral Red assay 19 h post exposure, i.e. 24 h after the start of the 5 h exposure, to cocaine (A), MDMA (B), methamphetamine (C), 3-MMC (D), and TFMPP (E) at 37 °C (black) or 41 °C (striped) ( $n_{\text{wells}} = 20-36$ ,  $N_{\text{plates}} = 4-6$ ). Cell viability is depicted as the mean  $\pm$  SEM (% of control). Effects  $\leq 10\%$  (i.e. the variation of vehicle control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. No biological relevant and significant differences were seen *versus* control and between temperatures. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**

Inhibition of neuronal activity by psychoactive substances at physiological (37 °C) or hyperthermic conditions (41 °C) compared to the estimated human brain concentration ([brain]). IC<sub>50</sub> values not within or close to the estimated human brain concentration are highlighted in grey. IC<sub>50</sub> values with 95% confidence intervals [CI] for the wMSR after acute (30 min) and prolonged exposure (4.5 h), and following recovery (19 h, i.e. 24 h after the start of the exposure) are shown. \* Indicates a significant difference ( $p < .05$ ) between 41 °C and 37 °C. <sup>a</sup>: Zwartsen et al. (2018), <sup>b</sup>: Hondebrink et al. (2018), <sup>c</sup>: Zwartsen et al. (2020).

Psychoactive drugs	Neuronal activity (wMSR) IC <sub>50</sub> values (μM)						[brain] (μM)
	37°C			41°C			
	Acute (30 min)	Prolonged (4.5 h)	Recovery (24 h post start exposure)	Acute (30 min)	Prolonged (4.5 h)	Recovery (24 h post start exposure)	
Cocaine	10 [9.2-11]	15 [10-29]	> 100	6.7 * [5.3-8.3]	14 [11-17]	> 100	0.02-30 <sup>a</sup>
MDMA	75 [63-88]	94 [77-121]	599 [325-1112]	41 * [33-54]	59 [36-192]	409 [231-743]	0.2-448 <sup>b</sup>
Methamphetamine	116 [84-143]	144 [98-182]	> 1000	57 * [41-91]	114 [58-168]	553 [238-1375]	0.05-56 <sup>a</sup>
3-MMC	59 [52-67]	76 [64-89]	545 [380-839]	30 * [22-43]	61 [50-75]	317 [215-487]	0.005-9.0 <sup>c</sup>
TFMPP	13 [11-15]	14 [11-19]	111 [57-255]	8.0 * [6.5-9.8]	15 [13-17]	103 [77-164]	22-89 <sup>b</sup>

exposure, recovery (19 h post exposure, i.e. 24 h after the start of the exposure) was slightly less pronounced following exposure at 41 °C. Additional research into the range of temperatures that can exacerbate effects on neuronal activity may ultimately reveal a threshold temperature that could be used for improving the hazard characterization of NPS and illicit drugs.

At 37 °C, cocaine, MDMA and TFMPP affected neuronal activity following acute and prolonged exposure at concentrations relevant for human exposure during recreational use, while methamphetamine and 3-MMC did not (Table 2). Following exposure at 41 °C, also methamphetamine affected neuronal activity at expected human brain concentrations. Our data and neuropathological and toxicological data of others (for reviews see Ginsberg and Busto (1998) and Kiyatkin (2005)), highlight that temperature is a critical factor influencing effects on neuronal function and should be considered in hazard characterization and risk assessment of psychoactive substances.

The mechanism(s) by which hyperthermia affects neuronal activity is currently unknown. However, hyperthermia affects many cellular processes that could influence neuronal activity. For instance, hyperthermia impairs energy metabolism and in turn reduces antioxidant defences (Skibba et al., 1991; Flanagan et al., 1998; Takeya, 2001; Dias da Silva et al., 2014; Valente et al., 2016a). In addition, *in vitro* studies showed exacerbation of cytotoxicity at hyperthermic conditions following exposure to high drug concentrations (Capela et al., 2006; Valente et al., 2016b), likely initiated by drug-induced ROS production (Dias da Silva et al., 2014; Valente et al., 2016b). In accordance with the absence of cytotoxicity in our experiments, cytotoxicity in those studies occurred only following (very) high concentrations and/or exposures exceeding 24 h, lacking human relevance.

An additional effect of hyperthermia-induced impaired energy metabolism is the accumulation of adenosine, a metabolite of the energy source adenosine triphosphate (ATP) (Takeya, 2001). Excess adenosine in turn reduces excitatory synaptic transmission by decreasing glutamate release (Motley and Collins, 1983; Dunwiddie, 1985; Flagmayer et al., 1997). Both ROS and adenosine accumulation may explain the decreased (reversibility of) neuronal activity at hyperthermic conditions vs. physiological temperatures (Beckhauser et al., 2016; Takeya, 2001).

While altered cellular processes at hyperthermic conditions may affect the reversibility of the drug-induced inhibition of neuronal activity, it is less likely involved in the exacerbation of the acute inhibition of neuronal activity following drug exposure as drug-induced effects were seen immediately following exposure. Additionally, the exacerbation is unlikely explained by the overall reduction of neuronal activity detected at 41 °C, as we previously showed that drug-induced inhibition of neuronal activity is independent of the activity during baseline (see Supplementary Fig. 1 in

Hondebrink et al. (2016)). Although difficult to prove, it is more plausible that the exacerbation of drug effects at 41 °C is caused by temperature-induced alterations in ion and receptor channel kinetics and dynamics, like the speed of ion channel opening and closing (Robertson and Money, 2012), the variation in total current passing through an open channel (Hille, 1978), and altered binding and gating properties of receptors (Postlethwaite et al., 2007; Millington et al., 2008; Gupta and Auerbach, 2011).

*In vivo*, the protective function of the blood-brain barrier (BBB) decreases at higher temperatures and following drug exposure (Sharma et al., 2009; Turowski and Kenny, 2015; Kiyatkin and Sharma, 2016). As the resulting impaired BBB could lead to higher brain concentrations of psychoactive drugs, the ~2-fold exacerbation of the neuronal effects at 41 °C that we observed in our *in vitro* model lacking a BBB could be even higher in patients.

In summary, exposure at hyperthermic conditions exacerbates the inhibition of neuronal activity following exposure to several psychoactive substances. This highlights the need to include temperature as a critical factor in future hazard assessment of illicit drugs and NPS and the need to closely monitor temperature of intoxicated patients.

## Funding

The work was supported by the Dutch Poisons Information Center (DPIC) (Utrecht Medical Center Utrecht, the Netherlands), and the Faculty of Veterinary Medicine (Utrecht University, the Netherlands).

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

We thank the members of the Neurotoxicology Research Group (Institute for Risk Assessment Sciences (IRAS), Utrecht University) for their helpful discussions.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.taap.2020.115015>.

## References

- Antia, U., Tingle, M.D., Russell, B.R., 2009. In vivo interactions between BZP and TFMPP (party pill drugs). *N Z Med J* 122, 29–38.
- Beckhauser, T.F., Francis-Oliveira, J., De Pasquale, R., 2016. Reactive oxygen species: physiological and physiopathological effects on synaptic plasticity. *Journal of Experimental Neuroscience* 10, 23–48.
- Capela, J.P., Ruscher, K., Lautenschlager, M., Freyer, D., Dirnagl, U., Gaio, A.R., Bastos, M.L., et al., 2006. Ecstasy-induced cell death in cortical neuronal cultures is serotonin 2A-receptor-dependent and potentiated under hyperthermia. *Neuroscience* 139, 1069–1081.
- Capela, J.P., Carmo, H., Remião, F., Bastos, M.L., Meisel, A., Carvalho, F., 2009. Molecular and cellular mechanisms of ecstasy-induced neurotoxicity: an overview. *Mol. Neurobiol.* 39, 210–271.
- Carvalho, M., Carmo, H., Costa, V.M., Capela, J.P., Pontes, H., Remião, F., Carvalho, F., et al., 2012. Toxicity of amphetamines: an update. *Arch. Toxicol.* 86, 1167–1231.
- Cruickshank, C.C., Dyer, K.R., 2009. A review of the clinical pharmacology of methamphetamine. *Addiction* 104, 1085–1099.
- Dias da Silva, D., Silva, E., Carmo, H., 2014. Combination effects of amphetamines under hyperthermia: the role played by oxidative stress. *J. Appl. Toxicol.* 34, 637–650.
- Dingemans, M.M., Schutte, M.G., Wiersma, D.M., de Groot, A., van Kleef, R.G., Wijnolts, F.M., Westerink, R.H., 2016. Chronic 14-day exposure to insecticides or methylmercury modulates neuronal activity in primary rat cortical cultures. *Neurotoxicology* 57, 194–202.
- Dunwiddie, T.V., 1985. The physiological role of adenosine in the central nervous system. *Int Rev Physiol* 27, 63–139.
- Flagmayer, I., Haas, H.L., Stevens, D.R., 1997. Adenosine A1 receptor-mediated depression of corticostriatal and thalamostriatal glutamatergic synaptic potentials in vitro. *Brain Res.* 778, 178–185.
- Flanagan, S.W., Moseley, P.L., Buettner, G.R., 1998. Increased flux of free radicals in cells subjected to hyperthermia: detection by electron paramagnetic resonance spin trapping. *FEBS Lett.* 431, 285–286.
- Ginsberg, M.D., Busto, R., 1998. Combating hyperthermia in acute stroke: a significant clinical concern. *Stroke* 29, 529–534.
- Glennon, R.A., 2014. Bath salts, mephedrone, and methylenedioxypyrovalerone as emerging illicit drugs that will need targeted therapeutic intervention. *Adv. Pharmacol.* 69, 581–620.
- Greene, S.L., Dargan, P.I., O'Connor, N., Jones, A.L., Kerins, M., 2003. Multiple toxicity from 3,4-methylenedioxymethamphetamine ("ecstasy"). *Am. J. Emerg. Med.* 21, 121–124.
- Greene, S.L., Kerr, F., Braitberg, G., 2008. Review article: amphetamines and related drugs of abuse. *Emerg Med Australas* 20, 391–402.
- Guatteo, E., Chung, K.K.H., Bowala, T.K., Bernardi, G., Mercuri, N.B., Lipski, J., 2005. Temperature sensitivity of dopaminergic neurons of the substantia nigra pars compacta: involvement of transient receptor potential channels. *J. Neurophysiol.* 94, 3069–3080.
- Gupta, S., Auerbach, A., 2011. Temperature dependence of acetylcholine receptor channels activated by different agonists. *Biophys. J.* 100, 895–903.
- Hille, B., 1978. Ionic channels in excitable membranes: current problems and biophysical approaches. *Biophys. J.* 22, 283–294.
- Hondebrink, L., Verboven, A.H.A., Drega, W.S., Schmeink, S., de Groot, M.W.G.D.M., van Kleef, R.G.D.M., Wijnolts, F.M.J., et al., 2016. Neurotoxicity screening of (illicit) drugs using novel methods for analysis of microelectrode array (MEA) recordings. *Neurotoxicology* 55, 1–9.
- Hondebrink, L., Zwartsen, A., Westerink, R.H.S., 2018. Effect fingerprinting of new psychoactive substances (NPS): what can we learn from in vitro data? *Pharmacol. Ther.* 182, 193–224.
- Johnstone, A.F.M., Gross, G.W., Weiss, D.G., Schroeder, O.H.U., Gramowski, A., Shafer, T.J., 2010. Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century. *Neurotoxicology* 31, 331–350.
- Jufer, R.A., Wstadik, A., Walsh, S.L., Levine, B.S., Cone, E.J., 2000. Elimination of cocaine and metabolites in plasma, saliva, and urine following repeated oral administration to human volunteers. *J. Anal. Toxicol.* 24, 467–477.
- Kalant, H., 2001. The pharmacology and toxicology of "ecstasy" (MDMA) and related drugs. *Cmaj* 165, 917–928.
- Kiyatkin, E.A., 2005. Brain hyperthermia as physiological and pathological phenomena. *Brain Res. Rev.* 50, 27–56.
- Kiyatkin, E.A., 2007. Brain temperature fluctuations during physiological and pathological conditions. *Eur. J. Appl. Physiol.* 101, 3–17.
- Kiyatkin, E.A., 2013. The hidden side of drug action: brain temperature changes induced by neuroactive drugs. *Psychopharmacology* 225, 765–780.
- Kiyatkin, E.A., Sharma, H.S., 2016. Breakdown of blood-brain and blood-spinal cord barriers during acute methamphetamine intoxication: role of brain temperature. *CNS Neurol Disord-DR* 15, 1129–1138.
- Millington, M., Bridle, H., Jesorka, A., Lincoln, P., Orwar, O., 2008. Ligand-specific temperature-dependent shifts in EC50 values for the GABAA receptor. *Anal. Chem.* 80, 340–343.
- Motley, S.J., Collins, G.G.S., 1983. Endogenous adenosine inhibits excitatory transmission in the rat olfactory cortex slice. *Neuropharmacology* 22, 1081–1089.
- Nakashima, K., Todd, M.M., 1996. Effects of hypothermia on the rate of excitatory amino acid release after ischemic depolarization. *Stroke* 27, 913–918.
- Nicolas, J., Hendriksen, P.J., van Kleef, R.G., de Groot, A., Bovee, T.F., Rietjens, I.M., Westerink, R.H., 2014. Detection of marine neurotoxins in food safety testing using a multi-electrode array. *Mol. Nutr. Food Res.* 58, 2369–2378.
- Postlethwaite, M., Hennig, M.H., Steinert, J.R., Graham, B.P., Forsythe, I.D., 2007. Acceleration of AMPA receptor kinetics underlies temperature-dependent changes in synaptic strength at the rat calyx of held. *J. Physiol.* 579, 69–84.
- Puia, G., Gullo, F., Dossi, E., Lecchi, M., Wanke, E., 2012. Novel modulatory effects of neurosteroids and benzodiazepines on excitatory and inhibitory neurons excitability: a multi-electrode array recording study. *Front Neural Circuit* 6, 94.
- Repetto, G., del Peso, A., Zurita, J.L., 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* 3, 1125–1131.
- Robertson, R.M., Money, T.G.A., 2012. Temperature and neuronal circuit function: compensation, tuning and tolerance. *Curr. Opin. Neurobiol.* 22, 724–734.
- Sharma, H.S., Muresanu, D., Sharma, A., Patnaik, R., 2009. Cocaine-induced breakdown of the blood-brain barrier and neurotoxicity. *Int. Rev. Neurobiol.* 88, 297–334.
- Shimshoni, J.A., Britzi, M., Sobol, E., Willenz, U., Nutt, D., Edery, N., 2015. 3-methylmethcathinone: pharmacokinetic profile evaluation in pigs in relation to pharmacodynamics. *J. Psychopharmacol.* 29, 734–743.
- Skibba, J.L., Powers, R.H., Stadnicka, A., Cullinane, D.W., Almagro, U.A., Kalbfleisch, J.H., 1991. Oxidative stress as a precursor to the irreversible hepatocellular injury caused by hyperthermia. *Int. J. Hyperther.* 7, 749–761.
- Strickland, J.D., Martin, M.T., Richard, A.M., Houck, K.A., Shafer, T.J., 2018. Screening the ToxCast phase II libraries for alterations in network function using cortical neurons grown on multi-well microelectrode array (mwMEA) plates. *Arch. Toxicol.* 92, 487–500.
- Takeya, M., 2001. Intrinsic factors involved in the depression of neuronal activity induced by temperature increase in rat hippocampal neurons. *Kurume Med J* 48, 295–306.
- Tukker, A.M., Wijnolts, F.M.J., de Groot, A., Westerink, R.H.S., 2018. Human iPSC-derived neuronal models for in vitro neurotoxicity assessment. *Neurotoxicology* 67, 215–225.
- Turowski, P., Kenny, B.-A., 2015. The blood-brain barrier and methamphetamine: open sesame? *Front. Neurosci.* 9, 156.
- Tyrkko, E., Andersson, M., Kronstrand, R., 2016. The toxicology of new psychoactive substances: synthetic cathinones and phenylethylamines. *Ther. Drug Monit.* 38, 190–216.
- UNODC, United Nations Office on Drugs and Crime, 2019a. World drug report 2019: Stimulants. [https://wdr.unodc.org/wdr2019/prelaunch/WDR19\\_Booklet\\_4\\_STIMULANTS.pdf](https://wdr.unodc.org/wdr2019/prelaunch/WDR19_Booklet_4_STIMULANTS.pdf).
- UNODC, United Nations Office on Drugs and Crime, 2019b. World drug report 2019: Global overview of drug demand and supply. [https://wdr.unodc.org/wdr2019/prelaunch/WDR19\\_Booklet\\_2\\_DRUG\\_DEMAND.pdf](https://wdr.unodc.org/wdr2019/prelaunch/WDR19_Booklet_2_DRUG_DEMAND.pdf).
- Valente, M.J., Araujo, A.M., de Lourdes Bastos, M., Fernandes, E., Carvalho, F., Guedes de Pinho, P., Carvalho, M., 2016a. Characterization of hepatotoxicity mechanisms triggered by designer cathinone drugs ( $\beta$ -keto amphetamines). *Toxicol. Sci.* 153, 89–102.
- Valente, M.J., Araujo, A.M., Silva, R., Bastos Mde, L., Carvalho, F., Guedes de Pinho, P., Carvalho, M., 2016b. 3,4-Methylenedioxypyrovalerone (MDPV): in vitro mechanisms of hepatotoxicity under normothermic and hyperthermic conditions. *Arch. Toxicol.* 90, 1959–1973.
- Vassallo, A., Chiappalone, M., De Camargos Lopes, R., Scelfo, B., Novellino, A., Defranchi, E., Palosaari, T., et al., 2017. A multi-laboratory evaluation of microelectrode array-based measurements of neural network activity for acute neurotoxicity testing. *Neurotoxicology* 60, 280–292.
- Volgushev, M., Kudryashov, I., Chistiakova, M., Mukovski, M., Niesmann, J., Eysel, U.T., 2004. Probability of transmitter release at neocortical synapses at different temperatures. *J. Neurophysiol.* 92, 212–220.
- Walter, E.J., Carraretto, M., 2016. The neurological and cognitive consequences of hyperthermia. *Critical Care (London, England)* 20, 199.
- Xie, T., McCann, U.D., Kim, S., Yuan, J., Ricaurte, G.A., 2000. Effect of temperature on dopamine transporter function and intracellular accumulation of methamphetamine: implications for methamphetamine-induced dopaminergic neurotoxicity. *J. Neurosci.* 20, 7838.
- Zwartsen, A., Hondebrink, L., Westerink, R.H., 2018. Neurotoxicity screening of new psychoactive substances (NPS): effects on neuronal activity in rat cortical cultures using microelectrode arrays (MEA). *Neurotoxicology* 66, 87–97.
- Zwartsen, A., Hondebrink, L., Westerink, R.H.S., 2019. 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. *Neurotoxicology* 74, 28–39.
- Zwartsen, A., Olijhoek, M.E., Westerink, R.H.S., Hondebrink, L., 2020. Hazard characterization of synthetic cathinones using viability, monoamine reuptake and neuronal activity assays. *Front. Neurosci.* 14, 9.