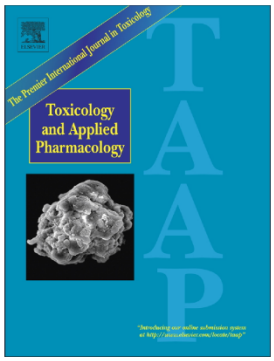

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Hyperthermia exacerbates the acute effects of psychoactive substances on neuronal activity measured using microelectrode arrays (MEAs) in rat primary cortical cultures *in vitro*

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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, and after 30 min and 4.5 h drug exposure (1-1000 µM) at 37°C or 41°C. Neuronal activity was also measured after a washout period of 19.5 h (24 h after the start of the exposure) at 37°C to investigate recovery of neuronal activity.

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).

Keywords: hyperthermia; designer drugs; hazard characterization; *in vitro* neuronal function; neuronal activity.

1. Introduction

One in twenty people between 15-64 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 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 Sprayt 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 µL cell suspension was added to each well of a 48-well MEA plate (1×10^5 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 µL dissection medium was added to each well. The day after cell plating (day *in vitro* 1; DIV1), 450 µL/well dissection medium was replaced with 450 µL/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 DIV 4, 450 µL/well glutamate medium was replaced with 450 µL/well NB-A FBS medium (glutamate free dissection medium).

For cytotoxicity measurements, 100 µL rat cortical cell suspension (3.0×10^4 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 µL/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_2/95% 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); (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 sec. 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_2/95% 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 µM, while MDMA, methamphetamine, and 3-MMC were tested at 10-1000 µM. 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 2-4 different isolations were used and tested in 3-5 plates (N_{plates}). The number of wells (n_{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;

$parameter_{exposure}$/$parameter_{baseline}$ 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 µM 3-MMC) and exposure scenario (acute, prolonged or recovery).

Outliers (>mean ± 2xSD) 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 mean ± SEM of n_{wells} from N_{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 2-3 different cultures) were exposed for 5 h at 41°C to cocaine, MDMA, methamphetamine, 3-MMC, and TFMPP (final concentrations 1-100 µM (cocaine and TFMPP) or 10-1000 µM (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% 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% H_2O, 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 µM 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 (> mean ± 2xSD; 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 mean ± SEM of n_{wells} from N_{plates}. For detailed information see Zwartsen *et al.* (2019); (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 IC_{50} values a four-parameter logistic curve with a variable slope was used (Y=Bottom+(Top-Bottom)/(1+10^((LogIC_{50}-X)*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 figure 3). To determine significant differences between temperatures at specific concentrations (multiple) unpaired T-tests were used (depicted by ^ in figure 3). Unpaired T-tests were also used to determine whether differences due to temperature in 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<0.05) and larger than the average variation of the control experiments (biological variation; ≥ 30% or ≥ 10%, for MEA and viability measurements respectively).

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=0.84). 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 ~10 µM, while IC_{50} values for the other drugs were 60-145 µM (Table 2). Neuronal activity increased to levels above baseline following recovery (19 h post exposure, *i.e.* 24 h after the start 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 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; Flagmeyer *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; Millingen *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.

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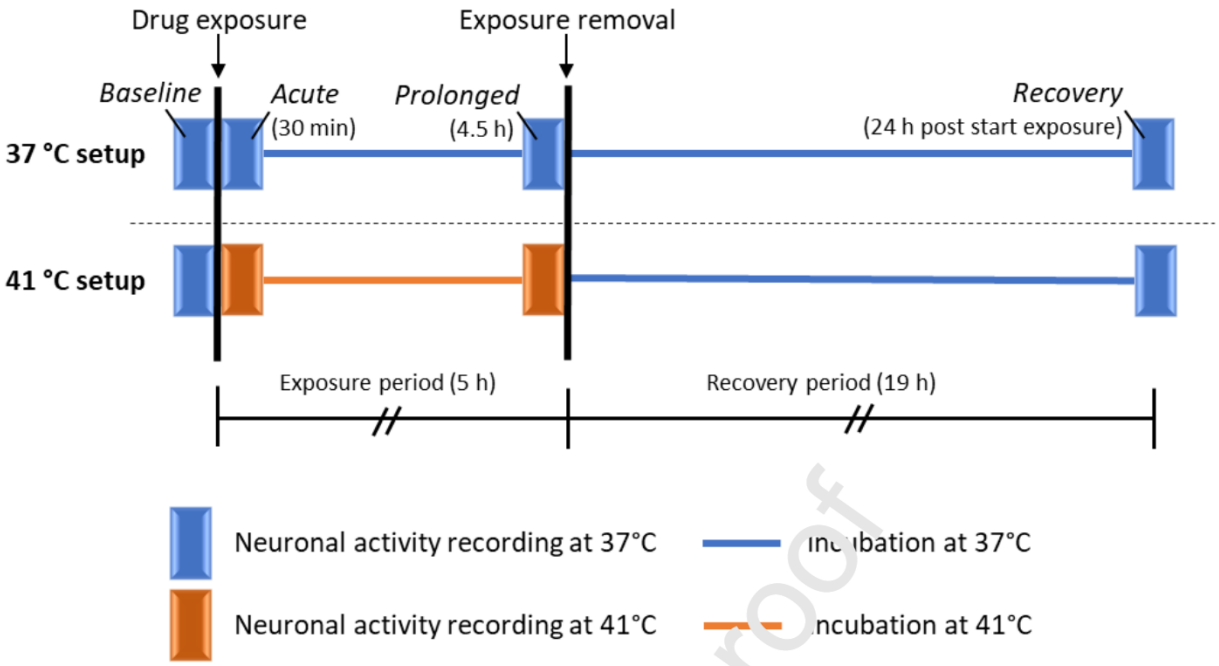
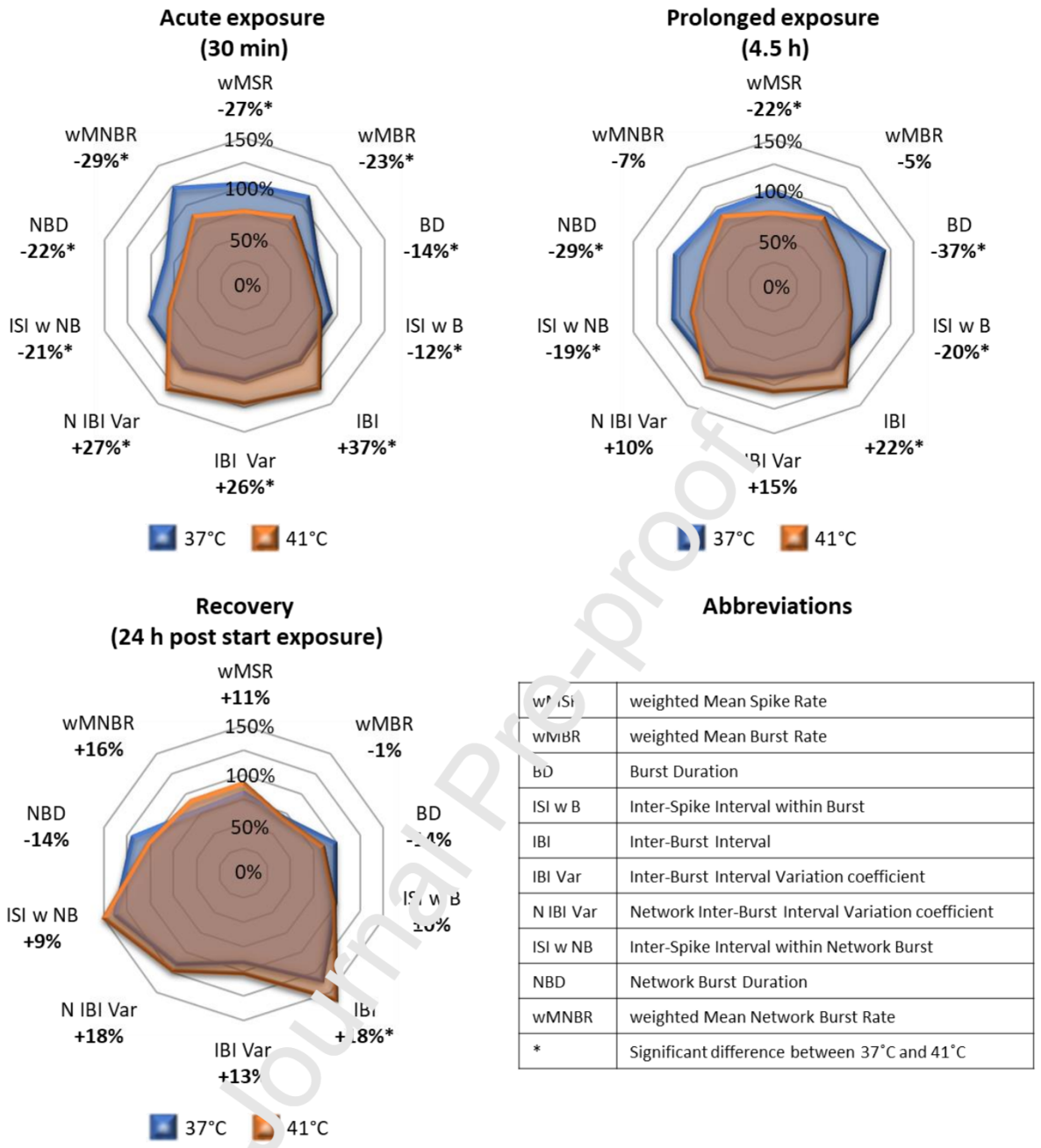


Fig. 1. Schematic illustration of recordings of neuronal activity at physiological (blue; 37°C) and hyperthermic temperatures (orange; 41°C).



Abbreviations

wMSR	weighted Mean Spike Rate
wMBR	weighted Mean Burst Rate
BD	Burst Duration
ISI w B	Inter-Spike Interval within Burst
IBI	Inter-Burst Interval
IBI Var	Inter-Burst Interval Variation coefficient
N IBI Var	Network Inter-Burst Interval Variation coefficient
ISI w NB	Inter-Spike Interval within Network Burst
NBD	Network Burst Duration
wMNBR	weighted Mean Network Burst Rate
*	Significant difference between 37˚C and 41˚C

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_{wells}= 73-90, N_{plates}= 26) and 41°C (n_{wells}= 108-131, N_{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<0.05). 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.

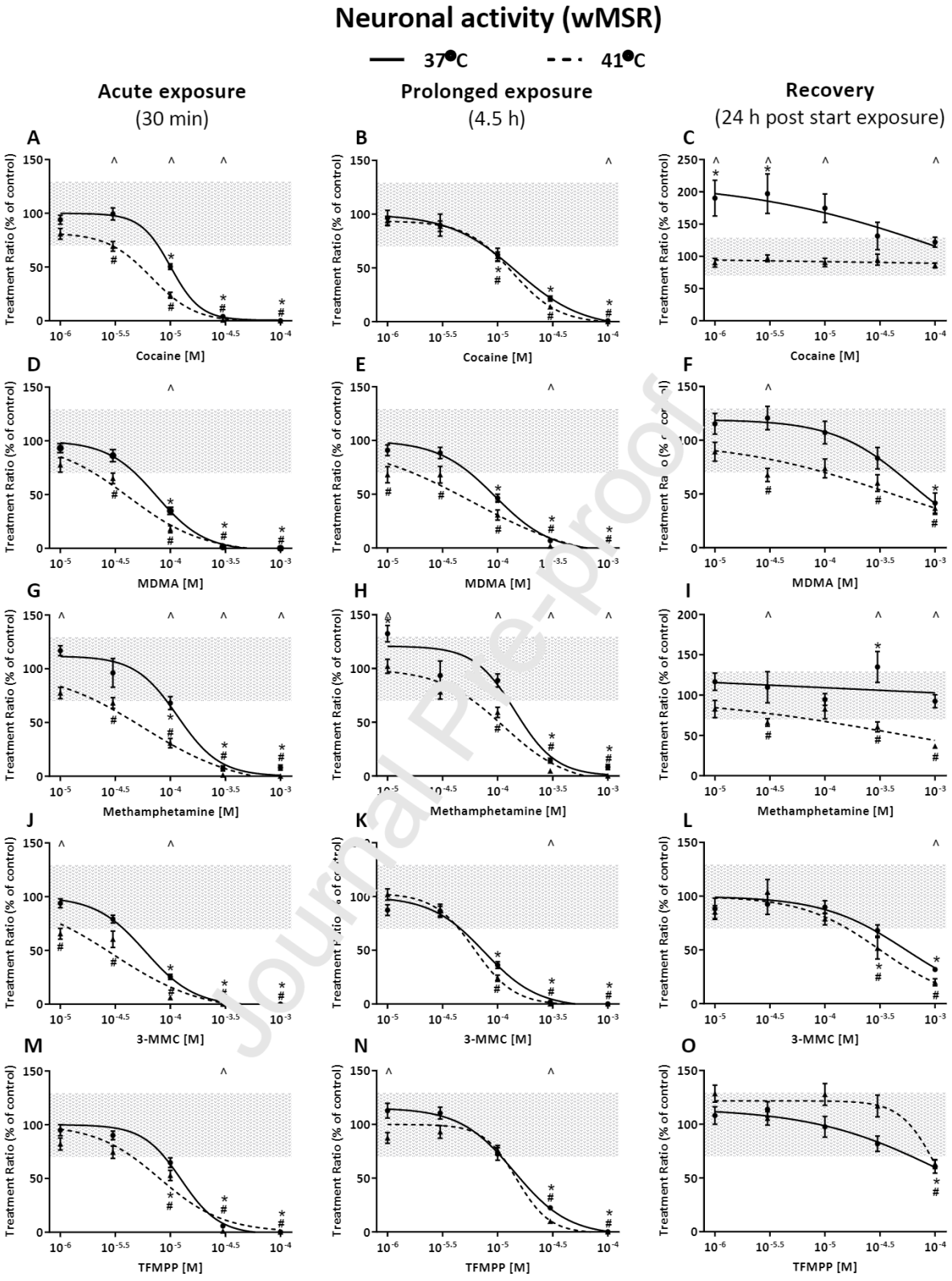


Fig. 3. Concentration-effect curves of psychoactive substances for neuronal activity at physiological (37°C; solid line) and hyperthermic (41°C; dashed line) conditions. The wMSR after acute (left; 30 min) and prolonged (middle; 4.5 h) exposure, and recovery (right; 24 h after the start of the exposure) are shown for cocaine (A-C), MDMA (D-F), methamphetamine (G-I), 3-MMC (J-L), and TFMPP (M-O) (n_{wells}= 8-34, N_{plates}= 3-7). Neuronal activity is depicted as the mean treatment ratio ±

SEM. Effects ≤ 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<0.05) are indicated with * for 37°C and with # for 41°C. ^ represents concentrations at which effects differed significantly between 37°C and 41°C.

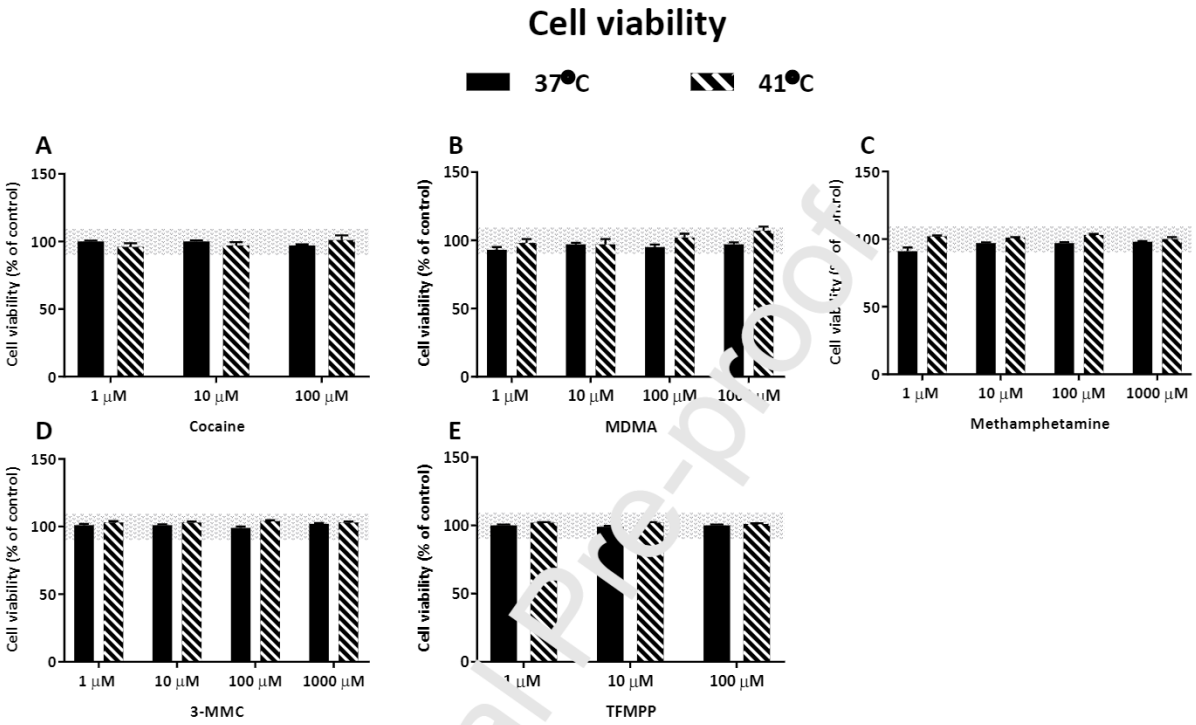


Fig. 4. Cell viability of rat cortical neurons after drug exposure at physiological (37°C; black) and hyperthermic (41°C; striped) conditions. Cytotoxicity was determined using a Neutral Red assay 19 h post exposure, *i.e.* 24h after the start of the 5 h exposure, to cocaine (A), MDMA (B), methamphetamine (C), 3-MMC (D), and TFMPP (E) at 37°C (black) or 41°C (striped) (n_{wells}= 20-36, N_{plates}= 4-6). Cell viability is depicted as the mean ± SEM (% of control). Effects ≤ 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.

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-ethylphenyl) 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-benzoloxy-8-methyl-8-azabicyclo[3.2.1] octane-4-carboxylate	50-36-2	> 98.5%	Spruyt Hillen

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_{50} values not within or close to the estimated human brain concentration are highlighted in grey. IC_{50} 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<0.05) between 41°C and 37°C. [a]: Zwartsen *et al.* (2018), [b]: Hondebrink *et al.* (2018), [c]: Zwartsen *et al.* (2020).

Psychoactive drugs	Neuronal activity (wMSR) IC_{50} 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 [10-19]	> 100	6.7 * [5.3-8.3]	14 [11-17]	> 100	0.02-30[a]
MDMA	75 [63-88]	94 [77-121]	599 [325-1112]	41 * [33-54]	59 [36-192]	409 [231-743]	0.2-448[b]
Meth-amphetamine	116 [84-143]	144 [98-182]	> 1000	57 * [41-91]	114 [58-168]	553 [238-1375]	0.05-56[a]
3-MMC	59 [52-67]	76 [64-89]	545 [380-839]	30 * [22-43]	61 [50-75]	317 [215-487]	0.005-9.0[c]
TFMPP	13 [11-15]	14 [11-19]	111 [57-255]	8.0 * [6.5-9.8]	15 [13-17]	103 [77-164]	22-89[b]

Author contributions

Anne Zwartsen: Conceptualization, Validation, Formal analysis, Investigation, Writing – Original Draft. **Laura Hondebrink**: Conceptualization, Writing – Review & Editing, Supervision. **Dylan de Lange**: Resources, Writing – Review & Editing, Funding acquisition. **Remco Westerink**: Conceptualization, Methodology, Writing – Review & Editing, Supervision

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Remco Westerink, on behave of all authors.

Highlights:

- In the absence of drugs, hyperthermic conditions decreased neuronal activity
- Psychoactive drugs inhibit neuronal activity at physiological temperatures
- At 41°C, drug-induced inhibition of neuronal activity was exacerbated
- Cell viability was unaffected following drug exposure at 37°C and 41°C
- Hyperthermic conditions should be included in neurotoxic hazard assessment

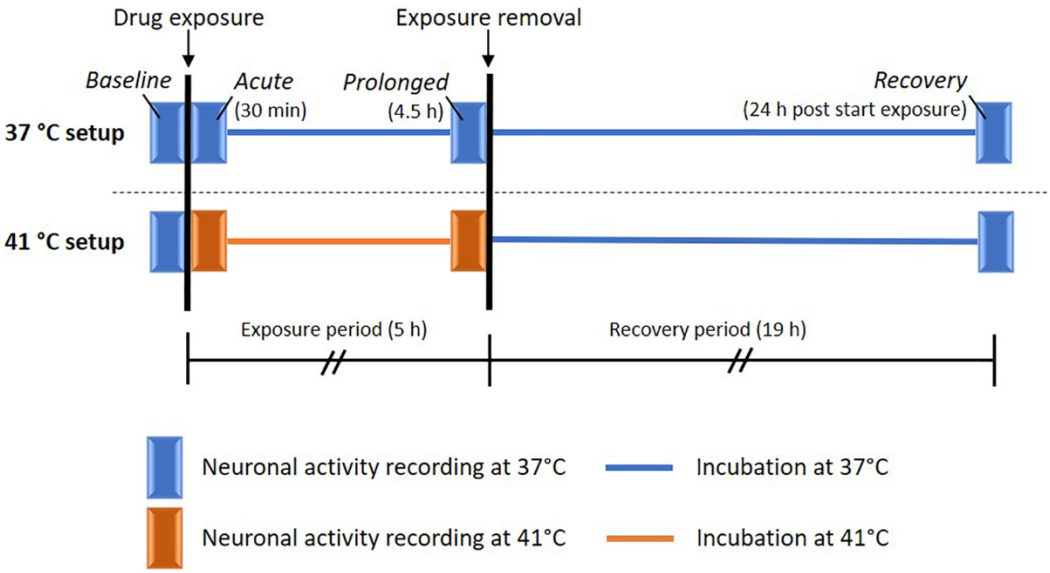
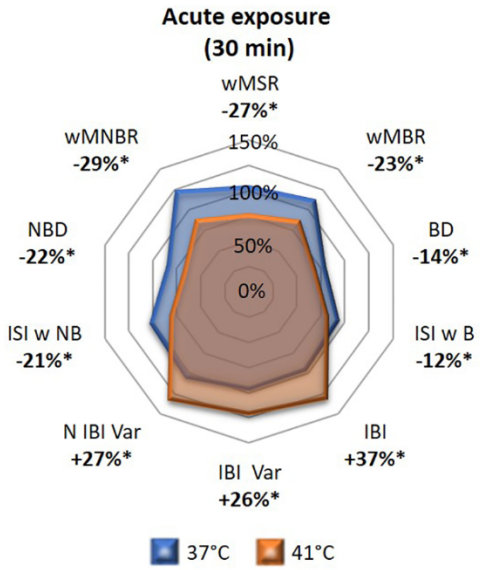
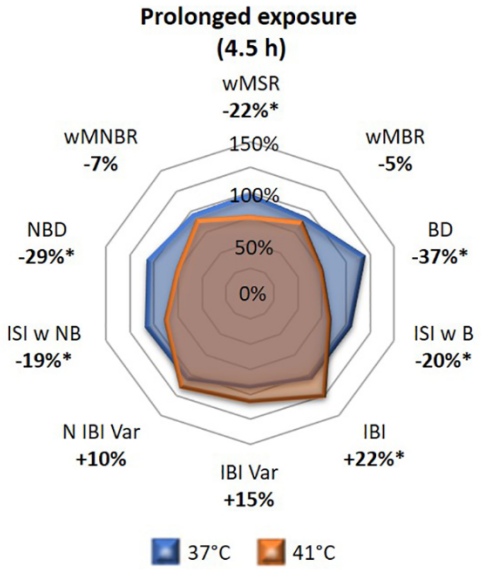
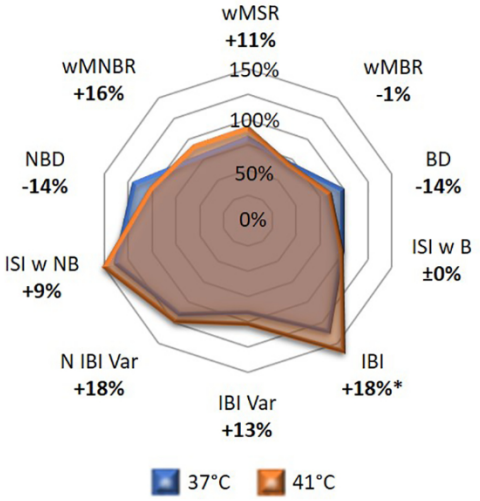

Drug exposure
Exposure removal
Baseline
Acute
(30 min)
Prolonged
(4.5 h)
Recovery
(24 h post start exposure)
37 °C setup
41 °C setup
Exposure period (5 h)
Recovery period (19 h)
Neuronal activity recording at 37°C
Incubation at 37°C
Neuronal activity recording at 41°C
Incubation at 41°C

Figure 1







Abbreviations

wMSR	weighted Mean Spike Rate
wMBR	weighted Mean Burst Rate
BD	Burst Duration
ISI w B	Inter-Spike Interval within Burst
IBI	Inter-Burst Interval
IBI Var	Inter-Burst Interval Variation coefficient
N IBI Var	Network Inter-Burst Interval Variation coefficient
ISI w NB	Inter-Spike Interval within Network Burst
NBD	Network Burst Duration
wMNBR	weighted Mean Network Burst Rate
*	Significant difference between 37˚C and 41˚C

Figure 2

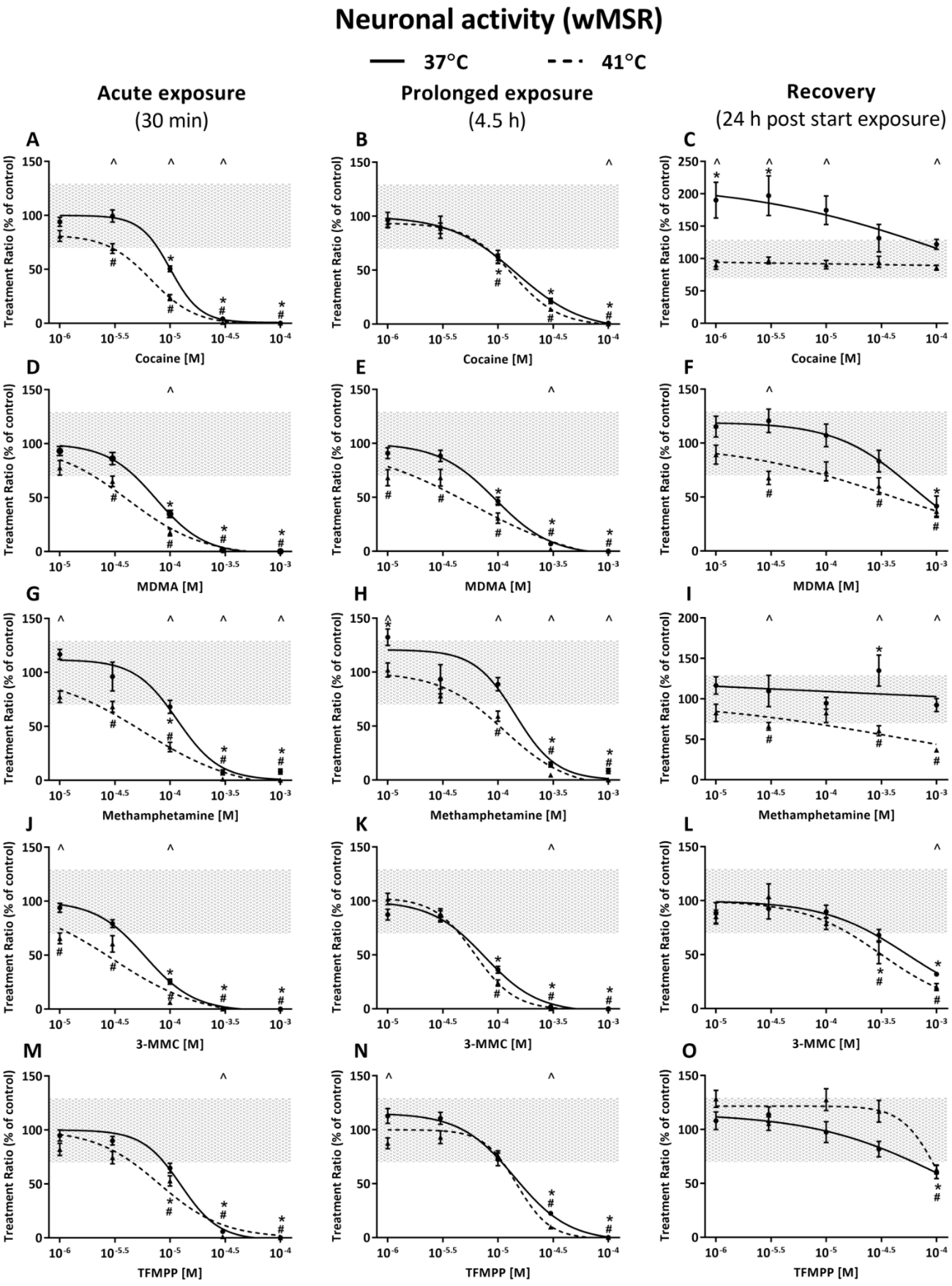

Neuronal activity (wMSR)
37°C
41°C
Acute exposure
(30 min)
Prolonged exposure
(4.5 h)
Recovery
(24 h post start exposure)
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
Treatment Ratio (% of control)
Cocaine [M]
MDMA [M]
Methamphetamine [M]
3-MMC [M]
TFMPP [M]

Figure 3

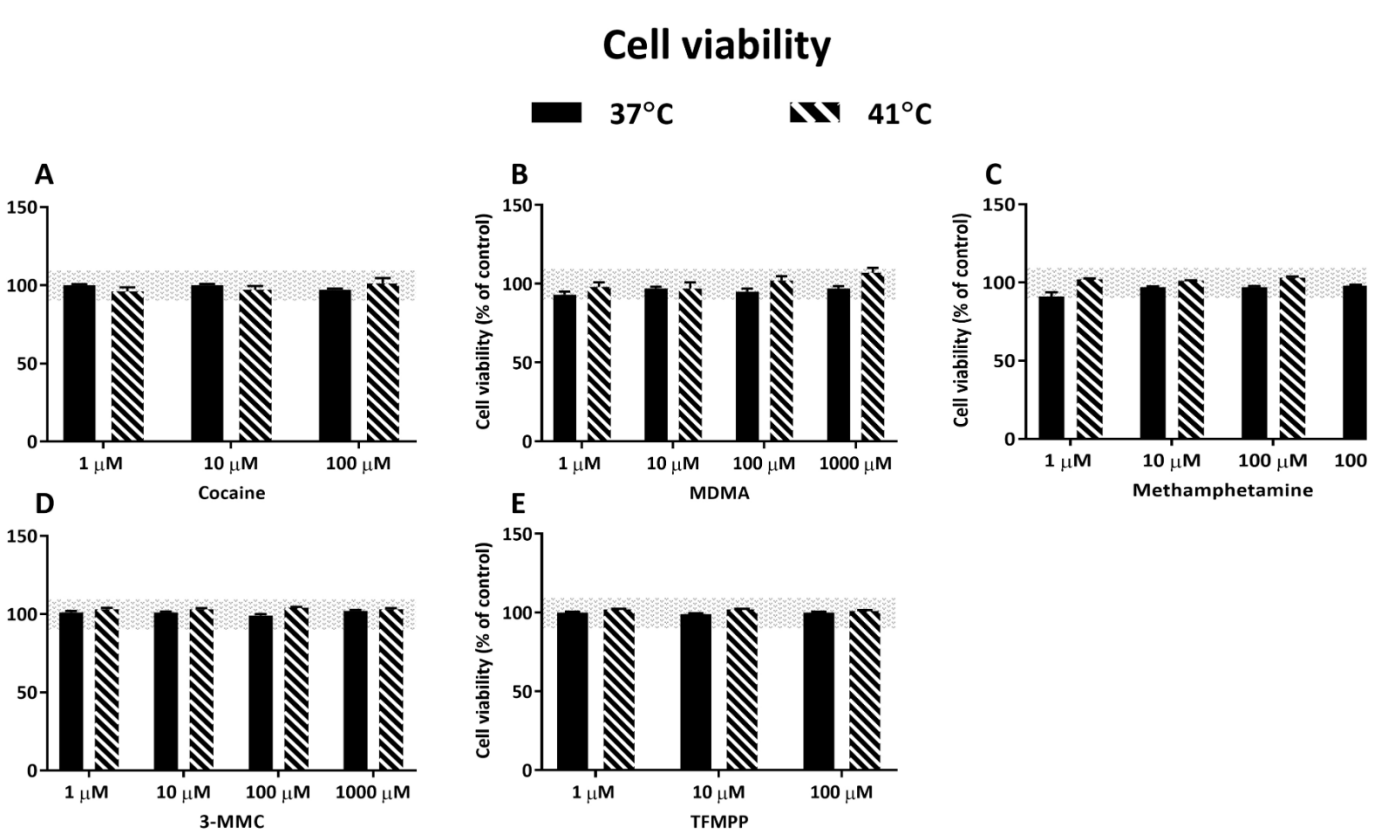

Cell viability
37°C
41°C
A
B
C
D
E
Cell viability (% of control)
150
100
50
0
1 μM
10 μM
100 μM
1000 μM
Cocaine
MDMA
Methamphetamine
3-MMC
TFMPP

Figure 4