Advancing cardiac safety and drug discovery screening using human stem cell-derived cardiomyocytes

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The use of recreational drugs, including new psychoactive substances (NPS), is paralleled by emergency department visits of drug users with severe cardiotoxicity. Drug-induced cardiotoxicity can be the (secondary) result of increased norepinephrine blood concentrations, but data on potential drug-induced direct effects on cardiomyocyte function are scarce. The presence of hundreds of NPS therefore calls for efficient screening models to assess direct cardiotoxicity.

We investigated effects of four reference compounds (3–30 nM dofetilide, nifedipine and isoproterenol, and 1–10 µM mexiletine) and six recreational drugs (0.01–100 µM cocaine, 0.01–1000 µM amphetamine, MDMA, 4- fluoroamphetamine, α-PVP and MDPV) on cardiomyocyte function (beat rate, spike amplitude and field potential duration (FPD ≈ QT interval in ECGs)), using Pluricyte® human-induced pluripotent stem cell (hiPSC)- derived cardiomyocytes cultured on ready-to-use CardioPlate™ multi-well microelectrode arrays (mwMEAs). Moreover, the effects of exposure to recreational drugs on cell viability were assessed.

Effects of reference compounds were in accordance with the literature, indicating the presence of hERG potassium (dofetilide), sodium (mexiletine) and calcium (nifedipine) channels and α-adrenergic receptors (isoproterenol). All recreational drugs decreased the spike amplitude at 10–100 µM. All amphetamine-type stimulants and α-PVP decreased the beat rate at 300 µM, while cocaine and MDPV did so at 10 µM and 30 µM, respectively. All drugs increased the FPD, however at varying concentrations. MDMA, MDPV and amphetamine affected cardiomyocyte function at concentrations relevant for human exposure, while other drugs affected cardiomyocyte function only at higher concentrations (≥ 10 µM). Cell viability was only mildly affected at concentrations well above the lowest concentrations affecting cardiomyocyte function.

We demonstrate that MEA recordings of hiPSC-derived cardiomyocytes enable screening for acute, direct effects on cardiomyocyte function. Our data further indicate that tachycardia in patients exposed to recreational drugs is likely due to indirect drug effects, while prolonged repolarization periods (prolonged QTc interval) could (partly) result from direct drug effects on cardiomyocyte function.

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Cardiotoxicity screening of illicit drugs and new psychoactive substances (NPS) in human iPSC-derived cardiomyocytes using microelectrode array (MEA) recordings

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Introduction

Approximately 5% of the general population has used a recreational drug at least once in their lifetime[1]. As an alternative to 'classic' illicit drugs, like cocaine, amphetamine and 3,4-methylenedioxymethamphetamine (MDMA), new psychoactive substances (NPS) entered the drug market around a decade ago[1]. Currently, there are over 700 of these 'legal highs' and the lifetime prevalence of NPS use is comparable to or even higher than that of 'classic' illicit drugs in specific groups, especially in young adults (15–24 years)[2]. NPS are manufactured to induce effects comparable to illicit drugs by modifying their chemical structures, thereby making them legal when entering the drug market in countries lacking an analogue and/or generic control system[3]. NPS of the stimulant (i.e. phenethylamines and synthetic cathinones) and synthetic cannabinoids class are most abundant and popular[1].

Exposure to illicit drugs can cause cardiovascular effects, ranging from mild tachycardia and hypertension to life-threatening arrhythmias and myocardial infarction. This has been well documented for 'classic' illicit drugs, but also for NPS, like cathinones and 4-fluoroamphetamine (4-FA)[4–10]. Due to the high number of NPS on the drug market, which potentially can cause severe cardiovascular effects, rapid cardiotoxicity screening of emerging NPS is essential. Current regulatory guidelines to investigate cardiotoxicity are based on whole animal *in vivo* studies and *in vitro* assays, using simplified cell lines overexpressing ion channels and receptors (e.g. the human ether-à-go-go-related gene (hERG) $K_v11.1$ K^+ channels), or primary cardiac tissues (animal-derived) to investigate drug-induced effects on cardiac function[11]. However, *in vivo* assays are low-throughput, labor intensive, costly and carry a heavy ethical concern. Moreover, although single target *in vitro* assays, such as the hERG channel assay, have been effective in preventing new proarrhythmic drugs from entering the market, their use could result in unnecessary retraction from the market of promising novel pharmaceuticals due to the lack of specificity. Not all hERG inhibitors prolong the QT interval or cause arrhythmias and it is now recognized that other ion channels could mitigate or abolish QT prolongation due to hERG channel blockage[12,13].

An integrated approach that allows for studying effects on multiple cardiac targets in a cell-based assay would be favorable for cardiotoxicity screening of new chemical entities and recreational drugs. Models that more closely resemble the complex electrophysiological properties of human cardiomyocytes would improve translation to the clinic and significantly contribute to the reduction, refinement and replacement of animal research (the 3Rs).

Recently, human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) have become available, providing a physiologically relevant cell model from an inexhaustible non-animal source, eliminating interspecies differences. Studies have shown that hiPSC-CMs express all major cardiac ion channels as seen in adult cardiomyocytes[14]. Although hiPSC-CMs often display fetal-like phenotypes (e.g. low expression of inward rectifying potassium channels or a more depolarized resting membrane potential[11,14,15]), they are widely being evaluated as

an alternative model for cardiac safety assessment, such as in the evolving Comprehensive *In vitro* Proarrhythmia Assay (CiPA) initiative[16]. Combined with appropriate functional techniques, hiPSC-CMs can serve as a physiologically relevant and integrated cell model to assess cardiotoxicity[15]. The gold-standard technique for measuring the electrical activity of hiPSC-CMs is the manual patchclamp technique. While this technique is highly sensitive, it is invasive, labor intensive, low-throughput, and uses single cells[17]. Novel hiPSC-CM studies, such as used within CiPA, focus on higher-throughput approaches like multielectrode arrays (MEA) or voltage-sensing optical (VSO) techniques to provide higher replicate numbers and reduced variability.

MEA allows for non-invasive and kinetic recording of extracellular field potentials of electrically coupled cardiomyocyte monolayers. Field potentials (FPs) correlate to action potentials typically measured using patch-clamp, and drug-induced effects correlate well to standard functional cardiac electrophysiology methods[18]. From these extracellular FP recordings, several parameters, including beat rate, field potential duration (FPD) and spike amplitude can be analyzed. These parameters are considered to resemble respectively heart rate, QT interval and QRS amplitude in an ECG. Drug-induced anomalies of these parameters can indicate cardiotoxicity, although additional assays are required to pinpoint the exact mechanism. Also, arrhythmia-like events, comparable to early afterdepolarizations and ectopic beats, can be detected. As a result, hiPSC-CMs combined with MEA recordings hold great potential to study drug-induced electrophysiological alterations and arrhythmias[16,19,20].

Illicit drugs affect cardiac function indirectly by increasing circulating norepinephrine levels through the inhibition of monoamine reuptake transporters[21,22]. Circulating norepinephrine activates peripheral adrenergic receptors, resulting in an increased heart rate and blood pressure[23]. In addition, drugs like cocaine can have a direct effect on cardiomyocyte function by blocking potassium, sodium and calcium channels. This can prolong the QT interval, subsequently introducing ventricular tachycardia called Torsades de Pointes (TdP), which can lead to sudden cardiac death[22,24]. Amphetamine and MDMA are thought to lack the ion channel-blocking properties of cocaine and induce tachycardia only indirectly via the sympathetic nervous system[25].

Currently, the mechanisms by which NPS affect cardiac function are not elucidated. NPS inhibit monoamine transporters[21], potentially leading to indirect cardiovascular effects. However, NPS may also have direct effects on cardiomyocyte function, irrespective of circulating norepinephrine levels. Human iPSC-CMs could be a suitable model to detect direct effects in the absence of norepinephrine. In this study, the functional presence of potential drug targets was investigated in hiPSC-CMs using MEA technology. After confirmation of the functional presence of these targets, as a proof-of-principle, we assessed whether recreational drugs directly affected cardiomyocyte function.

Results

Cardiomyocyte function

Cardiomyocyte function was investigated using pre-plated Pluricyte® hiPSC-derived Cardiomyocytes (Pluricyte® CardioPlate™ Maestro™ MEA 96) in combination with mwMEA recordings as an integrated testing strategy. The vehicle control wells of both cell batches, measured at two test locations, had an average beat period of 2.1 ± 0.04 s, a beat rate of 31 ± 0.6 beats/min, a spike amplitude of 2.4 ± 0.1 mV, a FPD of 0.9 ± 0.01 s and a FPD_c of 0.7 ± 0.01 s during the baseline recording (n_{wells}=80–83, N_{plates}=9. Minor changes between absolute values during baseline and exposure recording were observed in the vehicle control wells (Supplementary Table 1). Beat period, beat rate and $FPD_{(c)}$ were highly constant between wells and plates, while the spike amplitude was slightly dispersed most likely due to the differing proximity of the cells to the electrode[20]. See Supplementary Table 1 for baseline and exposure values for medium and DMSO-exposed vehicle controls.

Electrophysiological effect of reference compounds on cardiomyocyte function

Mexiletine was selected as a reference compound as it is a non-selective voltage-gated sodium channel blocker that reduces the upstroke velocity of the action potential, correlated to the spike amplitude of the field potential, and has hERG channel blocking properties. Exposure of hiPSC-CMs to 10 µM mexiletine, significantly decreased the spike amplitude by 70 ± 3.7%, decreased beat rate by 38 ± 4.2% and prolonged FPD_c by 23 ± 1.3%. It also resulted in quiescence in one-third of the wells (Figure. 2a and 3a Lab I; Supplementary Table 2). Nifedipine, selected as a reference compound for its calcium channel blocking effects, significantly reduced FPD_c (−11 ± 3.3%) at 30 nM, but had no significant effect on beat rate, spike amplitude or the number/percentage of active wells and electrodes (Figure. 2b and 3b Lab I; Supplementary Table 3). Dofetilide, an anti-arrhythmic drug and selective hERG-channel blocker with pro-arrhythmic potential, significantly increased the FPD_c (+27 ± 2.6%) and decreased the spike amplitude (−31 ± 9.9%) at 3 nM (Figure. 2c and 3c Lab I; Supplementary Table 4). It also induced flattening of the repolarization peak, quiescence, and arrhythmia in 17%, 17% and 17% of disparate wells, respectively. Isoproterenol, a β1- and β2-adrenergic receptor agonist used to treat bradycardia and heart block, had no effect on spike amplitude, but significantly increased the beat rate (+45 ± 7.7%) and decreased FPD_c (−28 ± 5.3%) at 30 nM (Figure. 2d and 3d Lab I; Supplementary Table 5). Effects of other concentrations of the reference compounds can be found in Supplementary Tables 2–5.

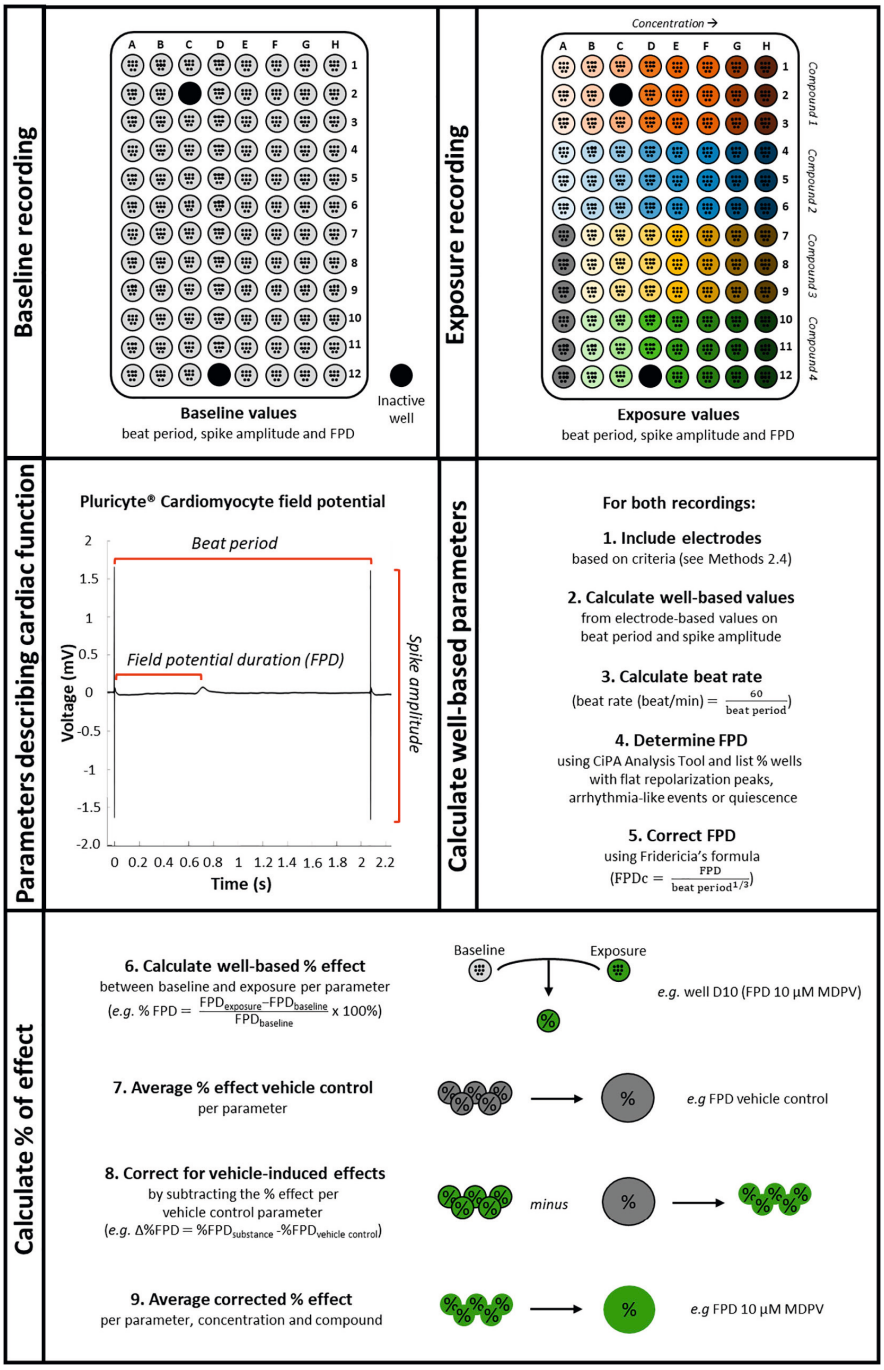

Figure 1. Schematic illustration of the data analysis from a Pluricyte® Cardiomyocyte field potential to the calculation of substance-induced effects on electrophysiological parameters. *For a detailed description see Section 2.4.*

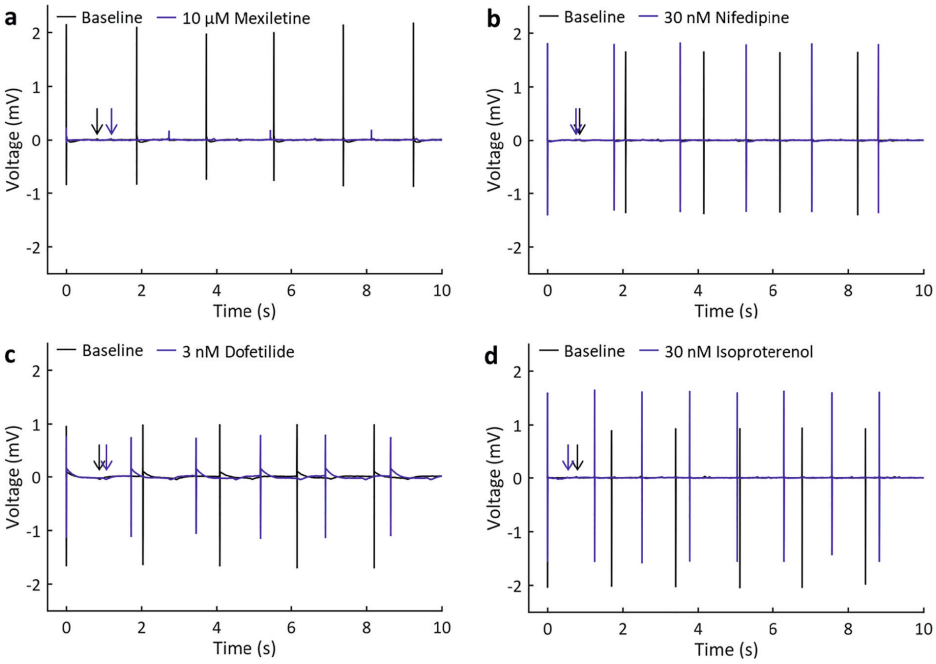

Figure 2. Beating pattern of cardiomyocytes before (baseline, black line) and after exposure to reference compounds (blue line). *Ten seconds from representative recordings are shown for (a) mexiletine, (b) nifedipine, (c) dofetilide and (d) isoproterenol. Arrows indicate the first repolarization peak. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

To determine the reproducibility of effects, reference compounds were measured at two sites. The effects observed for the various reference compounds were qualitatively comparable between the two test sites (Figure 3, Lab I and II), despite some differences in the magnitude of the effect for several parameters.

Electrophysiological effects of recreational drugs on cardiomyocyte function

Cocaine and amphetamine-type stimulants

Cocaine affected cardiomyocyte function at ≥ 10 µM: the beat rate and the spike amplitude decreased by 44% and 51%, respectively, and the $FPD_{(c)}$ was prolonged ≥ 2-fold (Figure. 4a and 5; Supplementary Table 6). At concentrations ≥ 30 µM, cocaine induced quiescence, resulting in loss of signal from electrodes and eventually from complete wells. Amphetamine-type stimulants (ATS) MDMA, amphetamine and 4-FA all decreased the spike amplitude at 100 µM (Figure. 4b–d and 6a–c; Supplementary Tables 7–9). Also, at 0.1 µM MDMA, yet not at higher concentrations, an increase in spike amplitude was seen. Ten micromolar amphetamine increased the beat rate by 20%, while at 300 µM all ATS decreased the beat rate varying between −28% to −82%. In addition, all ATS prolonged the FPD_c concentration-dependently, although with different potencies and effect sizes (Figure. 4b–d and 6a–c; Supplementary Tables 7–9). MDMA, amphetamine and 4-FA evoked quiescence at concentrations ≥ 300 µM.

Cathinones

At concentrations ≥ 10 µM, MDPV concentration-dependently decreased the spike amplitude. Moreover, MDPV reduced the beat rate at ≥ 30 µM (Figure. 7a and 8a; Supplementary Table 10). At 10 µM and 30 µM, MDPV prolonged the $FPD_{(c)}$. At higher concentrations, flattening of the repolarization peak and quiescence were seen. α-PVP also inhibited the spike amplitude in a concentration-dependent manner, but at concentrations 3-fold higher compared to MDPV (Figure 7b and 8b; Supplementary Table 11). Moreover, concentrations necessary to inhibit the beat rate or increase the $FPD_{(c)}$ were also 10-fold higher compared to MDPV. α-PVP induced quiescence starting from 300 µM.

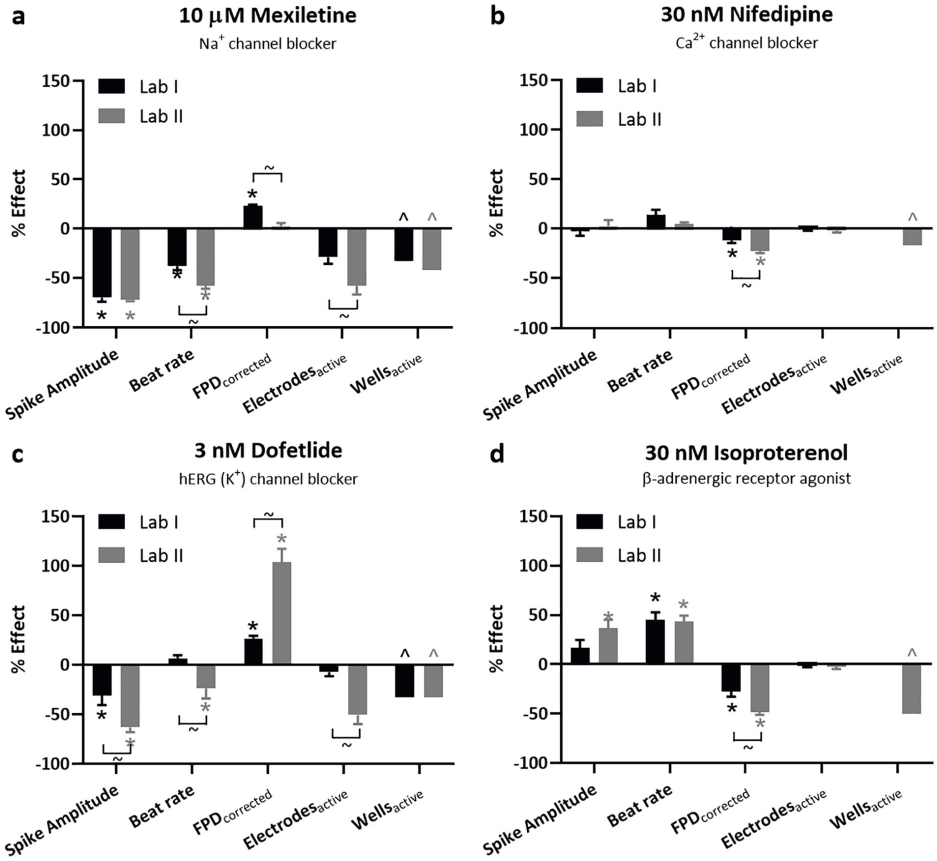

Figure 3. Effect of reference compounds on beat rate, spike amplitude, corrected field potential duration (FPDc), and the percentage of active wells and electrodes. *Effects are depicted as mean ± SEM (% change from baseline, corrected for vehicle control) of n_{wells} = 11–12, N_{plates} = 4 (Lab I, IRAS, Utrecht, The Netherlands) or n_{wells} = 12, N_{plates} = 3 (Lab II, Ncardia, Leiden, The Netherlands). * Effects significantly different from vehicle control (p < .05). ~ Values significantly different between laboratories (p < .05). See Supplementary Tables 2–5 for effects of other concentrations of reference compounds. ^ Mexiletine Lab I: 4/12 Q; Lab II: 4/12 IR, 1/12 A. ^ Nifedipine Lab II: 1/12 Q, 1/12 A. Dofetilide Lab I: 2/12 F, 2/12 Q, 2/12 A, Lab II: 1/12 Q, 3/12 A. Isoproterenol Lab II: 6/12 Q. Q (quiescence), F (flat repolarization peak), A (arrhythmia-like events), IR (irregular beating).*

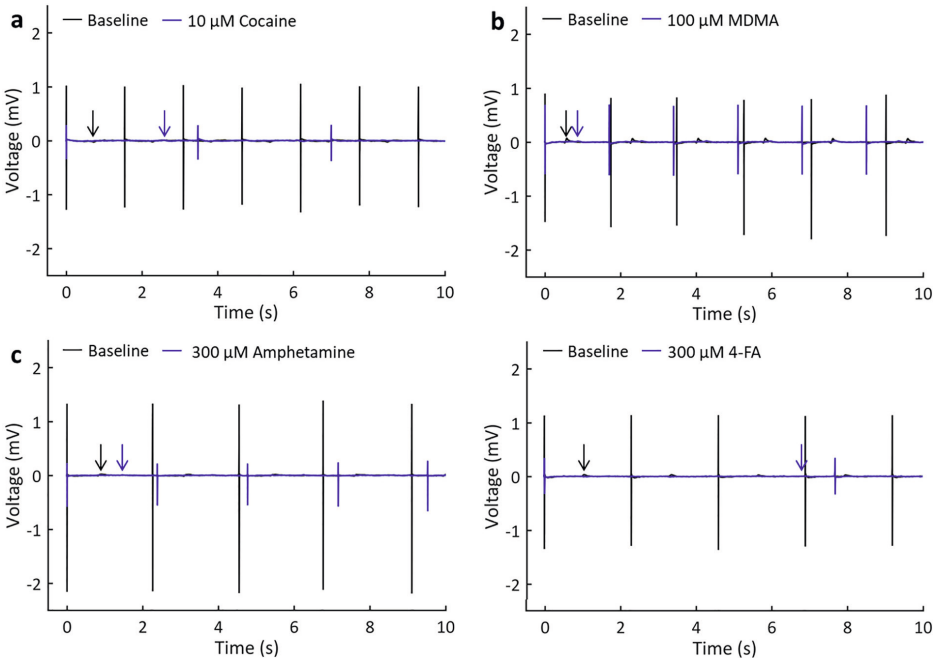

Figure 4. Beating pattern of cardiomyocytes before (baseline, black line) and after exposure to recreational drugs (blue line). *Ten seconds from representative recordings are shown for (a) cocaine, (b) MDMA, (c) amphetamine, and (d) 4-FA. Arrows indicate the first repolarization peak. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

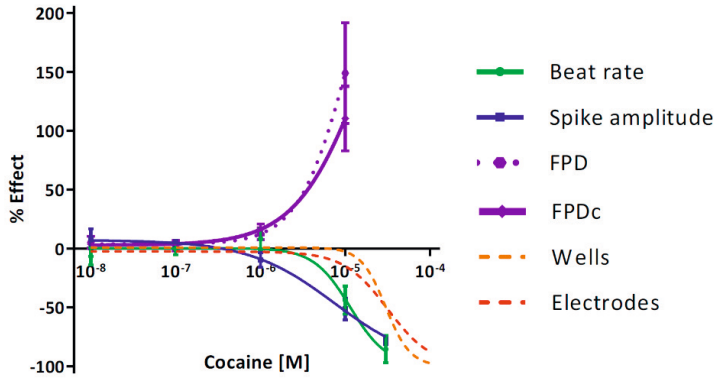

Figure 5. Concentration-effect curves of cocaine on cardiomyocyte function. *Effects on beat rate, spike amplitude and FPD(c) are shown for n wells = 5–6, Nplates = 3. Effects are depicted as the mean change±SEM from baseline, corrected for vehicle control. The % active wells and electrodes (in the remaining active wells) are shown by the dotted lines.*

Cell viability

Metabolic activity

To determine whether electrophysiological effects induced by the recreational drugs were due to changes in cell viability, the metabolic activity of the hiPSC-CMs was measured using the Alamar Blue assay directly after exposure (30 min; Figure 9a) and after a wash-out period of 23 h *(Figure 9b)*. A small decrease (~15%) in metabolic activity was seen directly after exposure to ≥ 300 µM amphetamine and 4-FA, and to 1000 µM MDMA. No changes in metabolic activity were present 24 h after exposure, except for the highest concentration of α-PVP, which decreased metabolic activity 24 h after exposure by 13% compared to control.

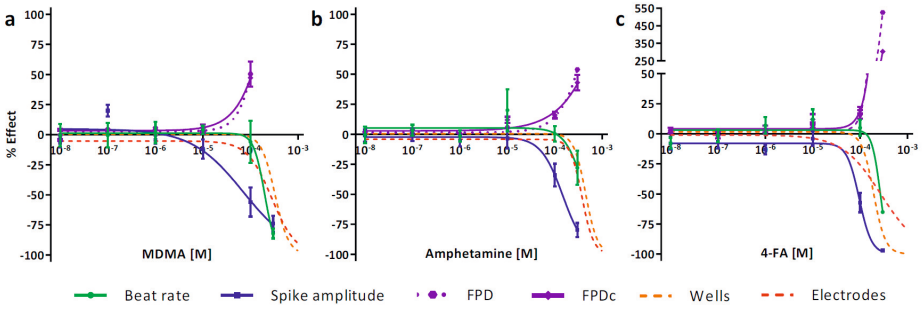

Figure 6. Concentration-effect curves of amphetamine-type stimulants (ATS) on cardiomyocyte function. *Effects on beat rate, spike amplitude and $FPD_{(c)}$ are shown for (a) MDMA, (b) amphetamine, and (c) 4-FA (n_{wells}=5–8, N_{plates}=3). Effects are depicted as the mean change ± SEM from baseline, corrected for vehicle control. Note the different scaling of the Y-axis for 4-FA in c. The percentage of active wells and electrodes (in the remaining active wells) are shown by the dotted lines. For exact values, statistically and biologically relevant effects, and the number of wells per condition, see Supplementary Tables 7–9.*

Active transport and lysosomal activity

Drug effects on cell viability were also determined by measuring active transport and lysosomal activity of hiPSC-CMs using the Neutral Red assay 24 h after the (30 min) drug exposure. All drugs were tested at the two highest concentrations at which the electrophysiological effects were determined. None of the drugs significantly affected cell viability *(Figure 10a)*.

Enzymatic activity and membrane integrity

Drug effects on cell viability were also determined by measuring enzymatic (esterase) activity and membrane integrity of hiPSC-CMs using the CFDA-AM assay 24 h following the (30 min) drug exposure. All drugs were tested at the two highest concentrations at which the electrophysiological effects were determined. Only 1000 µM amphetamine and 300 µM α-PVP induced a small, but significant, reduction of cell viability *(Figure 10b)*.

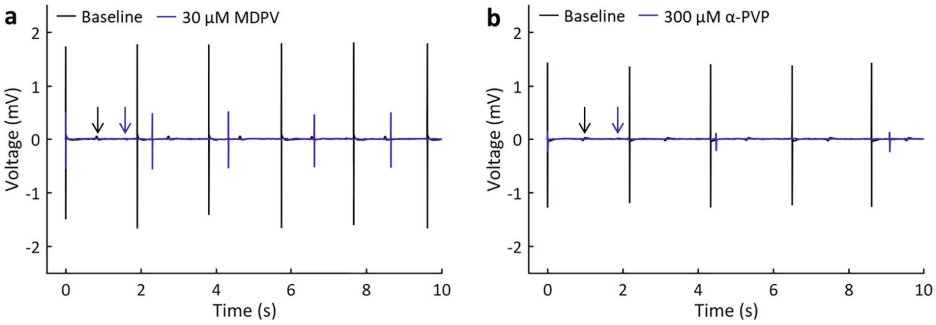

Figure 7. Beating pattern of cardiomyocytes before (baseline, black line) and after exposure to cathinones (blue line). *Ten seconds from representative recordings are shown for (a) MDPV and (b) α-PVP. Arrows indicate the first repolarization peak. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

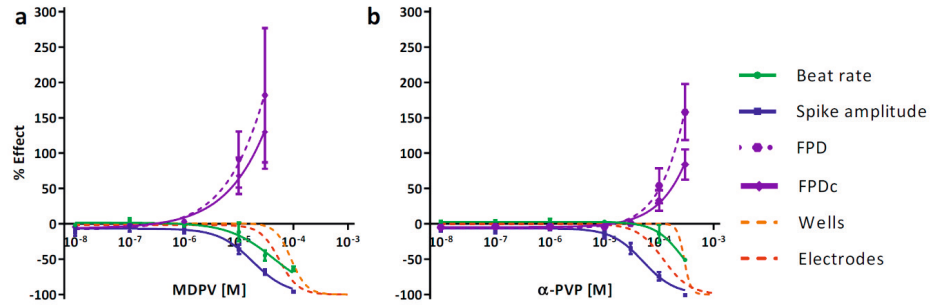

Figure 8. Concentration-effect curves of cathinones on cardiomyocyte function. *Effects on beat rate, spike amplitude and FPD(c) are shown for (a) MDPV and (b) α-PVP (nwells=7, Nplatex=3). Effects are depicted as the mean change±SEM from baseline, corrected for vehicle control. Effects on well and electrode activity (in the remaining active wells) are shown by the dotted lines. For exact values, statistically and biologically relevant effects, and the number of wells per condition, see Supplementary Tables 10–11.*

Discussion

Data on direct effects of recreational drugs on cardiomyocyte function are scarce and resources for a careful cardiotoxicity assessment of the many new NPS that enter the market annually are lacking. This highlights the need for novel, efficient screening assays to assess direct cardiotoxicity. The present study was conducted to investigate acute, direct effects of illicit drugs and NPS on cardiomyocyte function using hiPSC-CMs in combination with mwMEA technology.

To illustrate the functional presence of several known targets of recreational drugs in our *in vitro* model, effects of specific reference compounds targeting sodium, calcium and potassium (hERG) channels, and β-adrenergic receptors were included. These reference compounds

affected hiPSC-CM function by altering beat rate, spike amplitude and FPD(c), or by inducing arrhythmia-like events, in line with literature[16,18,28,32–34]. Our interlaboratory comparison showed that the effects of the reference compounds could be detected at both test sites. While differences in the magnitude of effects were seen, especially for the spike amplitude and FPD_c following dofetilide exposure, the nature of effects (inhibition or increase) was comparable between the two test sites *(Figure 3)*. Overall, it seems that hiPSC-CMs were more sensitive to the reference compounds at test site 2 (Ncardia, Leiden, The Netherlands) compared to test side 1 (Neurotoxicology group, Utrecht, The Netherlands). Differences may be related to shipment and shipping conditions, whether experiments were done in a CO_2 controlled environment, and/or to differences in assay day, laboratory, and operator.

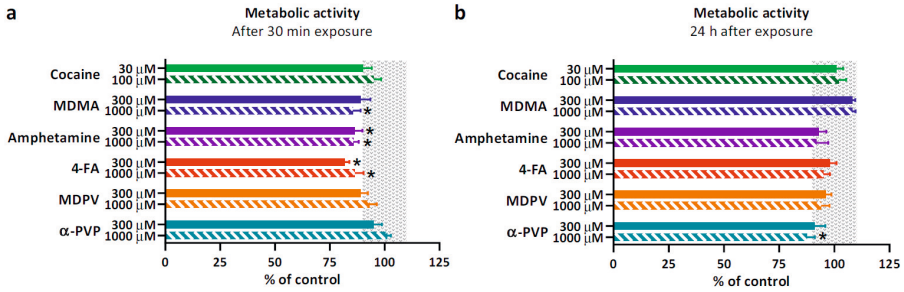

Figure 9. Cell viability of hiPSC-CMs following exposure to recreational drugs: effects on metabolic activity. *Metabolic activity was determined directly after 30 min exposure (a) and after a wash-out period of 23 h (b) following exposure using AlamarBlue assay. Effects of the two highest concentrations of cocaine, MDMA, amphetamine, 4-FA, MDPV, and α-PVP are depicted as the mean % of control ± SEM for nwells = 6–7, Nplates = 2. Effects ≤ 10% (i.e. the variation of control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects (> 10%, the variation of vehicle control) that are statistically different from control (p < .05) are indicated with *.*

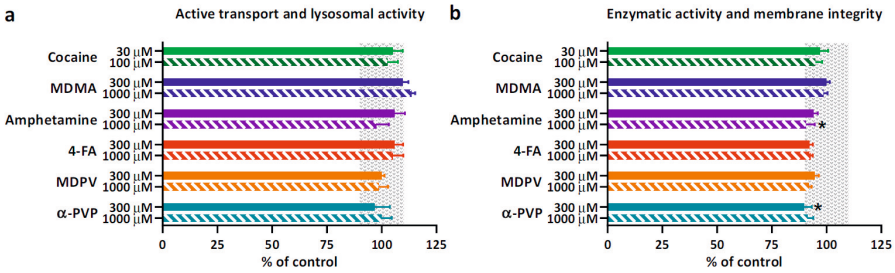

Figure 10. Cell viability of hiPSC-CMs following exposure to recreational drugs: effects on active transport and lysosomal activity (a), and enzymatic activity and membrane integrity (b). *Effects were determined after a wash-out period of 23 h following a 30 min exposure using the Neutral Red assay (a) and the CFDA-AM assay (b). Effects of the two highest concentrations of cocaine, MDMA, amphetamine, 4-FA, MDPV, and α-PVP are depicted as the mean % of control ± SEM for n_{wells}=6–7, N_{plates}=2. Effects ≤ 10% (i.e. the variation of control) are considered not to be of (toxicological) relevance, which is depicted by the grey area. Relevant effects (> 10%, the variation of vehicle control) that are statistically different from control (p < .05) are indicated with *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

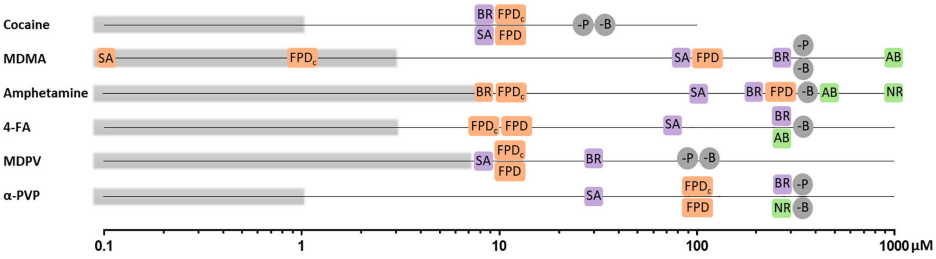

Figure 11. Schematic overview of the effects of illicit drugs and NPS on cardiomyocyte function. *Concentrations evoking the first significantly different effect from control (increase (orange) or decrease (purple)) on the beat rate (BR), spike amplitude (SA) or field potential duration$_{(corrected)}$ (FPD or FPD$_c$), are depicted for cocaine, MDMA, amphetamine, 4-FA, MDPV and α-PVP. In addition, the first concentration at which flattening of the repolarization peak (–P) or quiescence (–B) was seen is visualized. Also, the first concentration affecting cell viability is depicted in green (AB: AlamarBlue assay directly after 30 min exposure; NR: Neutral Red assay 24 h after 30 min exposure). Serum concentrations during recreational use are depicted by the grey areas. For serum concentrations see [21] (cocaine, MDMA, amphetamine, and 4-FA) and [38] (MDPV and α-PVP). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)*

All recreational drugs induced acute, concentration-dependent effects on one or more of the parameters indicative for electrophysiological dysfunction (Figure. 4–8, 11). All drugs affected the spike amplitude, beat rate and FPD$_{(c)}$. Prior to quiescence, an average reduction of −88% in spike amplitude was seen. All recreational drugs, except cocaine, inhibited the beat rate at concentrations 3–10-fold higher than the spike amplitude. For all drugs, increases in the FPD$_c$ ranging between +43% to +303% were seen at concentrations prior to flattening of the repolarization peak. The largest increase in FPD$_c$ was seen for 4-FA, however, this was only detected in a single well as all other wells at this concentration showed quiescence.

Cocaine is known to inhibit the potassium (hERG) channel at concentrations of 4–9 µM (IC$_{50}$; for review see[22]), block sodium channels from ≥10 µM[22,35] and activate L-type calcium channels at low concentrations (EC50: 274 nM[36]), while it inhibits L-type calcium channels at higher concentrations (≥ 30 µM[22]). We observed that cocaine prolonged the FPD$_c$ at 10 µM, as expected for an hERG channel blocker, and decreased the spike amplitude, most likely as a result of Na^+ channel blockade. While calcium channel blockade should be reflected by a decrease in FPD$_c$, cocaine blocks the hERG channel at a lower concentration, which most likely dominated the net outcome of the electrophysiological changes on the FPD$_c$.

We found no literature on direct effects of amphetamine-type stimulants on the electrophysiological function of cardiomyocytes *in vitro*, even though it has been suggested not to be of relevance (for review see[37]). Interestingly, our results do show acute, direct effects of MDMA, amphetamine and 4-FA on cardiomyocyte function (Figure. 6 and 11). While other targets may also play a role, the observed prolongation of the FPD$_{(c)}$ and the decrease in spike amplitude could indicate that hERG and sodium channels are targeted by amphetamine-type stimulants. The remarkable FPD$_c$ prolongation by 4-FA is of specific interest, as this substance is known to induce heart failure and Takotsubo cardiomyopathy[10]. As for amphetamine-type

stimulants, the cardiotoxic mechanisms of the cathinones MDPV and α-PVP are not yet elucidated. Our results show an acute, direct effect of both cathinones on the $FPD_{(c)}$, spike amplitude and beat rate of hiPSC-CMs. However, MDPV did so at concentrations 3–10-fold lower than α-PVP *(Figure 11)*. Future studies should be performed to determine the precise target(s) responsible for the observed drug-induced electrophysiological alterations. Such target identification assays can consist of (combinations of) biochemical methods, genetic interactions or computational models.

Recreational drug-induced changes in electrophysiological parameters were not biased by changes in cell viability after 30 min exposure. Changes in electrophysiological parameters were induced at concentrations well below concentrations that slightly decreased cell viability (< 15% decrease at the highest concentrations tested). To determine whether clinical effects associated with the use of these drugs could be due to direct cardiotoxic effects, effect concentrations were compared to the serum concentrations during recreational use *(Figure 11)*. Drug serum concentrations were gathered from literature reporting serum or blood drug concentrations of apprehended drivers, people arrested for criminal activity or partygoers[21,38]. At concentrations relevant for human recreational drug exposure, MDMA, MDPV and amphetamine directly affected cardiomyocyte function: MDMA prolonged the FPD_c, MDPV induced changes in spike amplitude and FPD_c, whereas amphetamine affected the beat rate and FPD_c. At slightly higher concentrations (~3 fold above recreational), 4-FA also directly affected cardiac function by increasing the FPD and FPD_c. In contrast, cocaine evoked direct cardiotoxic effects at concentrations ~10-fold above human recreational serum concentrations, whereas α-PVP evoked direct cardiotoxic effects at concentrations ~33-fold above human recreational serum concentrations. Moreover, at concentrations exceeding the recreational serum concentrations, all drugs will probably show acute, direct effects on cardiomyocyte function, possibly contributing to cardiac arrests and/or arrhythmias which is occasionally observed in drug poisoned patients. In addition, possible synergistic indirect or direct effects on cardiac function may occur due to multi-stimulant use[39].

At brain concentrations during recreational use, all drugs inhibit the norepinephrine reuptake transporter, resulting in higher concentrations of circulating norepinephrine and subsequent indirect effects on cardiac function[21,38]. The present research shows acute, direct effects on cardiac function, mostly on the repolarization period, at concentrations close to, or above, concentrations reached during recreational use. While the beating patterns of hiPSC-CMs cannot be compared directly to the human electrophysiology of the heart due to, for instance, a relatively immature phenotype, lack of various cell types and tissue structures, and lack of interaction with other organ systems, the cells do provide an elegant opportunity to study the direct effects of (potential) cardiotoxic substances. Our data shows mostly inhibitory effects of recreational drugs on the beat rate at concentrations above recreational serum concentrations. This suggests that tachycardia seen in clinical situations is unlikely due to acute, direct effects of any of these drugs at recreational doses, but rather results from indirect effects, such as an increase in blood norepinephrine levels. However, prolonged QT_c interval in drug-exposed

patients can be related to the increase in FPD_c seen in the current study and could be (partly) due to direct drug effects. In addition, cardiotoxic effects, like myocardial infarction, observed during overdoses could be related to the acute, direct effects of recreational drugs on cardiomyocyte function seen at top concentrations tested in the current study.

Research on the functional effects of recreational drugs is mostly focused on neuropharmacological and neurotoxic effects, although cardiotoxic effects are just as prominently seen in the clinic. In addition to neuropharmacological endpoints, effects on cardiomyocyte function should be investigated to assess the risks of illicit drugs and NPS. This study is to our knowledge the first to investigate and elucidate the acute, direct effects of NPS and illicit drugs (with the exception of cocaine) on hiPSC-derived cardiomyocyte function. Our results not only highlighted the applicability of hiPSC-CMs cultured on mwMEA plates as a screening tool for acute, direct effects of illicit drugs and NPS on cardiomyocyte function, but also confirmed the potency of recreational drugs to directly affect acute cardiomyocyte functioning without affecting cell viability.

Materials and methods

Chemicals

Reference compounds mexiletine, nifedipine, dofetilide, and isoproterenol were obtained from Bio-Techne Ltd. (Abingdon, United Kingdom). Stock solutions of the reference compounds (10 mM) were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich). At the day of exposure, reference compound stocks were freshly diluted in Pluricyte® Cardiomyocyte Medium (Ncardia, Leiden, The Netherlands) with a final concentration of ≤ 0.3% DMSO. The following concentrations were tested: 1, 3 and 10 µM (mexiletine) and 3, 10 and 30 nM (dofetilide, nifedipine and isoproterenol).

Cocaine (methyl (1R,2R,3S,5S)-3-benzoyloxy-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate) hydrochloride and DL-amphetamine (1-phenylpropan-2-amine) sulfate salts (purity>98.5%) were obtained from Spruyt Hillen IJsselstein, the Netherlands). MDMA ((RS)-1-(1,3-benzodioxol-5-yl)-N-methylpropan-2-amine), 4-FA (4-fluoroamphetamine; 1-(4-fluorophenyl)propan-2-amine), MDPV (1-(1,3- benzodioxol-5-yl)-2-pyrrolidin-1-ylpentan-1-one) and α-PVP (1-phenyl- 2-pyrrolidin-1-ylpentan-1-one) hydrochloride salts (purity>98.5%) were purchased from Lipomed (Weil am Rhein, Germany). At the day of exposure, stock solutions (10 mM) of NPS and illicit drugs were made in Pluricyte® Cardiomyocyte Medium and further diluted to exposure concentrations. The following concentrations were tested: 0.01, 0.1, 1, 10, 30, 100 µM (cocaine), 0.01, 0.1, 1, 10, 100, 300, 1000 µM (amphetamine, MDMA and 4-FA) and 0.01, 0.1, 1, 10, 30, 100, 300, 1000 µM (MDPV and α-PVP). Tested concentrations were based on concentrations relevant for human exposure (see Figure 11). Control wells were exposed to Pluricyte® Cardiomyocyte Medium

with (vehicle controls for reference compounds) or without (controls for recreational drugs) equimolar DMSO concentrations.

Human-induced pluripotent stem cell-derived cardiomyocytes

Ready-to-use Pluricyte® CardioPlate™ Maestro MEA96 plates were provided by Ncardia (Gosselies, Belgium). These CardioPlates™ are 96- well classic, mwMEA plates with 8 electrodes/well (Axion Biosystems, Inc., Atlanta, USA) pre-seeded with Pluricyte® Cardiomyocytes. Pluricyte® Cardiomyocytes are ventricular-enriched cardiomyocytes differentiated from hiPSCs without genetic modification or selection procedures. Cryopreserved Pluricyte® Cardiomyocytes were thawed, plated on the mwMEA plates and maintained for 7 days using Ncardia's standardized cell culture protocol. The CardioPlates™ were prepared for temperature-controlled shipment after appropriate quality control at day 7 post-plating. Upon arrival, the CardioPlate™ was immediately transferred to the incubator (37 °C, 5% CO2) for 30 min. Subsequently, the sealing mat was carefully removed under sterile conditions, the medium was changed using pre-warmed (37 °C) Pluricyte® Cardiomyocyte Medium (100 µL/well), and the CardioPlate™ was transferred back into the incubator (37 °C, 5% CO2) with a sterile lid. MEA recordings were performed at days 8–10 post-plating using two batches of hiPSC-CMs from different differentiations.

MEA recordings

At day 8–10 post-plating, 2 h after a medium change, Pluricyte® CardioPlate™ MEA plates were placed in the Maestro MEA device (768- channel amplifier) with an integrated heating system, temperature controller and data acquisition interface (Axion BioSystems, Inc., Atlanta, USA) to record spontaneous beating of the hiPSC-CMs. Pluricyte® CardioPlate™ MEA plates were allowed to equilibrate for 2 min prior to the start of a 5 min baseline recording (Cardiac Standard filters and amplifiers in spontaneous cardiac mode (12.5 Hz sampling frequency, 2 kHz Kaiser Window, 0.1 Hz IIR) at 37 °C). Following the baseline recording, all wells were exposed by manually pipetting 11 µL Pluricyte® Cardiomyocyte Medium with or without the test substances or vehicle (for final test concentrations see Section 2.1). To prevent possible effects of cumulative dosing, including receptor (de)sensitization, each well was exposed to only a single substance at a single concentration. After an equilibration period of 2 min, a 40 min recording was started to determine the acute, direct effects of the reference compounds, illicit drugs and NPS on spontaneously beating hiPSC-CMs. A minimum of 3 plates and 5 wells were used for each substance and concentration (for exact numbers see Supplementary Tables 1–11). For interlaboratory comparison, reference compounds were investigated both at the Neurotoxicology Research Group (Institute for Risk Assessment Sciences, Utrecht University, The Netherlands; Lab I) and Ncardia (Leiden, The Netherlands; Lab II). Illicit drugs and NPS were not tested at both locations, as specific permits are needed which were not available at Ncardia, Leiden. The same cell type, culture media, protocols, and reference compounds were used at both test sites.

Data analysis and statistics

Raw data files were re-recorded using the AxIS digital filters (Butterworth: 0.1 Hz (high) and 2 kHz (low)), cardiac beat detector (threshold: 300 µV, beat period: 800 ms (min), 5 s (max), inflection search (detection Auto (Max/Min), hold off: 100 ms (post), 50 ms (pre)) and statistic compiler (30 stable beats selection, no FPD quality control).

We analyzed electrode-based data of the beat period, spike amplitude and FPD for a 2 min window during baseline (baseline t = 3–5 min (t = 5)) and exposure recordings (two time windows were selected based on strongest effects: exposure t = 8–10 min (t = 10; reference compounds) and t = 28–30 min (t = 30; recreational drugs)). In addition, beat period data was used to calculate the beat rate in beats per min (beats/min = 60/beat period).

First, for all parameters, quiescent electrodes in the baseline recording, defined as no beats in the stable beat region (30 stable beats) of the selected time window, were excluded from further analysis *(Figure 1)*. Electrodes that were active (i.e. electrodes with ≥ 1 detected beat(s) in the stable beat region) during baseline recording, but showed quiescence or arrhythmia-like events during exposure recording, were excluded from further analysis, since the beat period, spike amplitude and FPD cannot be determined under these circumstances. The number of wells in which quiescence or arrhythmia-like events occurred and the percentage of active electrodes were reported. Subsequently, electrode-based values of the beat period, spike amplitude and beat rate were averaged per well for the baseline (t = 5) and exposure (t = 10 and t = 30) recordings to obtain well-based values *(Figure 1)*.

To determine the effects of the test substances on the FPD with high certainty, data files were analyzed using the CiPA Analysis Tool (Axion Biosystems, version 2.0.14). For each well, a 'golden electrode', i.e. an electrode with a trackable repolarization feature in both the baseline and exposure recording (matched), was selected and the markers to define the FPD were set manually when needed[26]. As such, well-based FPD values from baseline and exposure recordings (t = 10 or t = 30 min) were determined based on one representative electrode per well, since FPD values across electrodes from the same well were highly homogenous (data not shown). To correct the FPD for effects on the beat period, we used Fridericia's formula (Figure 1; FPD/beat period$^{1/3}$[27]). While not thoroughly validated for hiPSC-CMs, this formula is widely used in similar *in vitro* assays[16,28].

Subsequently, well-based values (based on all active electrodes) were used to determine substance or vehicle-induced effects on all parameters by calculating the percentage change between the baseline and the exposure measurements at either t = 10 or t = 30 min following exposure (Figure 1; paired comparison; e.g. $\%\ FPD_{t=10} = ((FPD_{exposure\ t=10} - FPD_{baseline})/FPD_{baseline})\times 100\%$). Thereafter, for each well, the average percentage change for the (cell-) batch-matched vehicle control wells is subtracted (e.g. $\Delta\%FPD_{t=10} = \%\ FPD_{substance\ t=10} - \%FPD_{vehicle\ control\ t=10}$). After outlier analysis, the percentage change (% effect) is averaged across replicates of a substance and concentration. Values were considered outliers when ≥

2xSD or ≤ 2xSD. In addition, the number of wells and electrodes/well that show quiescent, flat (no repolarization peak detected) or arrhythmic signals after exposure were expressed as a percentage of the number of active electrodes/well prior to exposure *(Figure 1)*.

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 and a one-way ANOVA followed by a Dunnet's post-hoc test was used to test for differences between concentrations and control, and experimental locations. Data are shown as mean ± SEM for n_{wells}, $N_{plate(s)}$. Relevant effects were defined as effects above the biological variation (SD) of the vehicle control wells (13% for the spike amplitude and beat rate, 7% for the FPD, and 5% for the FPD_c), and significantly different from the batch-matched vehicle control ($p < .05$). Traces were plotted using the Cardiac Data Plotting Tool (version 1.3.1, Axion Biosystems).

Cytotoxicity assays

To determine cell viability, ready-to-use clear bottom black polystyrene 96-well plates pre-seeded with Pluricyte® Cardiomyocytes (Ncardia, Gosselies, Belgium) were exposed to the two highest concentrations of each recreational drug (30 µM and 100 µM for cocaine; 300 µM and 1000 µM for MDMA, amphetamine, 4-FA, MDPV and **α**- PVP) or medium (vehicle) at day 8 post-plating.

hiPSC-CMs were exposed for 30 min, comparable to the 30 min exposure to determine electrophysiological effects. Metabolic activity, often a first sign of cell stress and/or reduced cell viability, was determined after 30 min with the AlamarBlue (AB) assay[29]. The exposure medium was replaced by 100 µL/well AB solution (25 µM AB (Invitrogen, Breda, The Netherlands) in phosphate-buffered saline (PBS 1×)). Following 45 min incubation in the dark at 37 °C, fluorescence was measured using a Tecan Infinite M1000 plate reader equipped with a 10W Xenon flashlight source (Tecan Group LtD; Männedorf, Switzerland). Fluorescence was measured spectrophotometrically at 540 nm excitation and 590 nm emission. Data were processed using iControl software (version 7.01). Following the AB assay, the plates were incubated for 23 h with fresh Pluricyte® Cardiomyocyte medium at 37 °C and 5% CO_2.

Next, 24 h post the 30 min exposure, using a combined AB and CFDA-AM assay, cell viability was measured again. In addition to metabolic activity (AB assay), enzymatic activity and membrane integrity were determined using the CFDA-AM assay. To do so, the medium was replaced with a 100 µL AB/CFDA-AM solution per well (25 µM AB and 10 µM CFDA-AM (Invitrogen, Breda, The Netherlands) in PBS[29]). Following 45 min incubation in the dark at 37 °C, fluorescence was measured at 540/590 nm (AB) 493/541 nm (CFDA-AM). Thereafter, the AB/CFDA-AM solution was removed and 100 µL Neutral Red (NR) solution (Invitrogen, Breda, The Netherlands; 12 µM in PBS) was added to each well to test for active transport and lysosomal activity[30] as a readout for cell viability. 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. Plates, covered in aluminium foil, were placed on a plate shaker for approximately 30 min

before fluorescence was measured at 530/645 nm. Cell viability was calculated according to Zwartsen, Hondebrink[30,31] and expressed as mean ± SEM of n_{wells} from N_{plates}. In total, 3.3% of the values were considered outliers. Data were processed using GraphPad Prism software and significance was determined using one-way ANOVA's followed by a Dunnet's post-hoc test. Relevant effects were defined as effects above the biological variation (SD) of the vehicle control wells (10%), and significantly different from the vehicle control ($p < .05$).

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Declaration of competing interest

Zwartsen, Westerink, de Lange and Hondebrink declare no conflicts of interest. Nacken and de Korte are employed by Ncardia, which is the supplier of the Pluricyte® CardioPlate™.

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Supplementary material

Supplementary Table I. hiPSC-derived cardiomyocyte function in control wells. Average absolute values of baseline and vehicle (medium or DMSO) exposure measurements and the mean percentage change ± SEM (% effect) between baseline and vehicle (medium or DMSO) exposure measurements are listed for several parameters: beat period (s), beat rate (beats/min), spike amplitude (mV), and the (corrected) field potential duration ($FPD_{(c)}$) (s) (see methods 2.4 for details). Data were measured using two batches of hiPSC-CMs at two test sites: Lab I, IRAS, Utrecht, The Netherlands (Table a, b) and Lab II, Ncardia, Leiden, The Netherlands (Table c).

a Lab I	Recreational drugs control (Medium vehicle, t= 30 min)			
	Batch I (n_{wells} =25, N_{plates} = 4)		Batch II (n_{wells} =12-13, N_{plates} = 2)	
	Baseline	Exposure	Baseline	Exposure
Beat period (s)	2.1 ± 0.07	1.7 ± 0.03	2.2 ± 0.05	2.3 ± 0.04
Beat rate (beats/min)	30 ± 0.8	35 ± 0.5	28 ± 0.6	26 ± 0.4
Spike amplitude (mV)	2.4 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	3.1 ± 0.2
FPD (s)	0.8 ± 0.02	0.8 ± 0.02	1.0 ± 0.02	1.3 ± 0.02
FPD_c (s)	0.7 ± 0.01	0.7 ± 0.01	0.8 ± 0.01	1.0 ± 0.01

b Lab I	Reference compounds control (DMSO vehicle, t= 10 min)			
	Batch I* (n_{wells} = 11-12, N_{plates} = 4)		Batch II (n_{wells} = 2, N_{plates} = 1)	
	Baseline	Exposure	Baseline	Exposure
Beat period (s)	1.9 ± 0.10	1.7 ± 0.06	2.3 ± 0.08	2.3 ± 0.08
Beat rate (beats/min)	33 ± 1.9	35 ± 1.2	26 ± 0.9	26 ± 1.0
Spike amplitude (mV)	2.5 ± 0.2	2.5 ± 0.2	2.8 ± 0.6	3.2 ± 0.6
FPD (s)	0.8 ± 0.02	0.8 ± 0.02	1.1 ± 0.03	1.2 ± 0.05
FPD_c (s)	0.6 ± 0.01	0.6 ± 0.01	0.8 ± 0.01	0.9 ± 0.03

c Lab II	Reference compounds control (DMSO vehicle, t= 10 min)			
	Batch I (n_{wells} = 7-8, N_{plate} = 1)		Batch II (n_{wells} = 10-11, N_{plates} = 2)	
	Baseline	Exposure	Baseline	Exposure
Beat period (s)	1.7 ± 0.06	1.7 ± 0.06	2.5 ± 0.10	2.5 ± 0.2
Beat rate (beats/min)	35 ± 1.3	36 ± 1.2	24 ± 0.9	25 ± 0.9
Spike amplitude (mV)	2.6 ± 0.2	2.6 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
FPD (s)	0.8 ± 0.02	0.8 ± 0.02	1.0 ± 0.03	1.0 ± 0.03
FPD_c (s)	0.7 ± 0.01	0.7 ± 0.01	0.8 ± 0.01	0.8 ± 0.02

* Only acute baseline and exposure values are depicted for 0.1% DMSO vehicle control wells, however reference compounds from Lab I, batch I were corrected with matched DMSO controls (0.03% (n_{wells} = 6, N_{plates} = 2), 0.1% or 0.3% (n_{wells} = 6, N_{plates} = 2)).

Supplementary Table II. hiPSC-derived cardiomyocyte function: concentration-effect data of mexiletine. The mean percentage change ± SEM between baseline and mexiletine exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Data were measured at two test sites: Lab I, IRAS, Utrecht, The Netherlands (Table a) and Lab II, Ncardia, Leiden, The Netherlands (Table b). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control.

a Lab I	**Mexiletine concentration (μM) (t= 10 min)**		
	1	**3**	**10**
Spike amplitude	-7.8 ± 2.8	-20 ± 2.9	-70 ± 3.7
Beat rate	-9.5 ± 3.0	-16 ± 1.9	-38 ± 4.2
FPD	+7.2 ± 2.0	+16 ± 1.7	+40 ± 2.2
FPD_c	+4.5 ± 1.2	+9.2 ± 1.2	+23 ± 1.3
Wells active	12/12	12/12	8/12^
Electrodes active	100% ± 0	99% ± 1.2	71% ± 6.6
n_{wells}, N_{plates}	n= 12, N= 4	n= 12, N= 4	n= 12, N= 4

^: 4/12 quiescence.

b Lab II	**Mexiletine concentration (μM) (t= 10 min)**		
	1	**3**	**10**
Spike amplitude	-4.8 ± 6.1	-38 ± 2.9	-71 ± 1.3
Beat rate	-5.3 ± 2.3	-22 ± 1.4	-58 ± 3.1
FPD	+2.0 ± 1.0	+13 ± 1.4	+31 ± 6.6
FPD_c	+1.5 ± 0.8	+4.9 ± 0.9	+2.5 ± 3.2
Wells active	11/12^	12/12	7/12^^
Electrodes active	94% ± 3.6	81% ± 6.5	42% ± 8.8
n_{wells}, N_{plates}	n= 12, N= 3	n= 12, N= 3	n= 12, N= 3

^: 1/12 inactive in baseline recording; ^^: 4/12 irregular beating, 1/12 arrhythmia-like events.

Supplementary Table III. hiPSC-derived cardiomyocyte function: concentration-effect data of nifedipine. The mean percentage change ± SEM between baseline and nifedipine exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Data were measured at two test sites: Lab I, IRAS, Utrecht, The Netherlands (Table a) and Lab II, Ncardia, Leiden, The Netherlands (Table b). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control.

a Lab I	Nifedipine concentration (nM) (t= 10 min)		
	3	10	30
Spike amplitude	+0.7 ± 3.3	+3.4 ± 3.1	-0.6 ± 4.1
Beat rate	-2.6 ± 1.8	+5.8 ± 3.3	+11 ± 5.7
FPD	+1.4 ± 2.2	-6.6 ± 3.1	-12 ± 4.3
FPD_c	+0.5 ± 1.9	-6.1 ± 2.6	-11 ± 3.3
Wells active	11/11^	11/11	11/11
Electrodes active	100% ± 0	96% ± 2.1	99% ± 1.1
n_{wells}, N_{plates}	n= 11, N= 4	n= 11, N= 4	n= 11, N= 4

^: 1/11 flat repolarization peak.

b Lab II	Nifedipine concentration (nM) (t= 10 min)		
	3	10	30
Spike amplitude	-1.8 ± 2.6	+14 ± 2.4	+2.3 ± 6.0
Beat rate	+4.9 ± 2.7	+4.8 ± 2.4	+4.5 ± 1.6
FPD	-4.4 ± 0.7	-13 ± 2.5	-25 ± 2.1
FPD_c	-3.5 ± 0.7	-9.7 ± 1.5	-23 ± 2.2
Wells active	12/12	11/12^	10/12^^
Electrodes active	97% ± 1.8	100% ± 0	98% ± 2.0
n_{wells}, N_{plates}	n= 12, N= 3	n= 12, N= 3	n= 12, N= 3

^: 1/12 arrhythmia-like events; ^^: 1/12 quiescence, 1/12 arrhythmia-like events.

Supplementary Table IV. hiPSC-derived cardiomyocyte function: concentration-effect data of dofetilide. The mean percentage change ± SEM between baseline and dofetilide exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Data were measured at two test sites: Lab I, IRAS, Utrecht, The Netherlands (Table a) and Lab II, Ncardia, Leiden, The Netherlands (Table b). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. -B: no beats/quiescence, and -P: flat repolarization peak.

a Lab I	Dofetilide concentration (nM) (t= 10 min)		
	3	10	30
Spike amplitude	-31 ± 9.9	-16 ± 4.8	-59 ± 22
Beat rate	+6.6 ± 3.1	+26 ± 16	+52 ± 19
FPD	+23 ± 3.8	+42 ± 4.1	-P
FPD_c	+27 ± 2.6	+52 ± 9.2	-P
Wells active	8/12^	3/12^^	3/12^^^
Electrodes active	93% ± 4.2	95% ± 4.8	45% ± 28
Data from n_{wells}, N_{plates}	n= 12, N= 4	n= 12, N= 4	n= 12, N= 4

^: 2/12 flat repolarization peak, 2/12 quiescence, 2/12 arrhythmia-like events; ^^: 5/12 quiescence, 4/12 arrhythmia-like events; ^^^: 3/12 flat repolarization peak, 8/12 quiescence, 1/12 arrhythmia-like events.

b Lab II	Dofetilide concentration (nM) (t= 10 min)		
	3	10	10
Spike amplitude	-63 ± 4.8	-70	-B
Beat rate	-24 ± 10	-66	-B
FPD	+145 ± 17	-P	-B
FPD_c	+104 ± 14	-P	-B
Wells active	8/12^	1/12^^	0/12^^
Electrodes active	49% ± 9.1	50%	-
n_{wells}, N_{plates}	n= 12, N= 3	n= 12, N= 3	n= 12, N= 3

^: 1/12 quiescence, 3/12 arrhythmia-like events; ^^: 1/12 inactive in baseline recording, 1/12 flat repolarization peak, 4/12 quiescence, 6/12 arrhythmia-like events; ^^^: 9/12 quiescence, 3/12 arrhythmia-like events.

Supplementary Table V. hiPSC-derived cardiomyocyte function: concentration-effect data of isoproterenol. The mean percentage change ± SEM between baseline and nifedipine exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Data were measured at two test sites: Lab I, IRAS, Utrecht, The Netherlands (Table a) and Lab II, Ncardia, Leiden, The Netherlands (Table b). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control.

a Lab I	Isoproterenol concentration (nM) (t= 10 min)		
	3	10	30
Spike amplitude	+2.0 ± 3.4	-0.5 ± 5.7	+17 ± 8.0
Beat rate	+11 ± 5.6	+41 ± 7.5	+45 ± 7.7
FPD	+4.4 ± 3.8	-26 ± 5.1	-34 ± 5.3
FPD_c	-2.7 ± 3.0	-18 ± 5.1	-28 ± 5.3
Wells active	12/12	12/12	12/12
Electrodes active	99% ± 1.2	94% ± 2.9	99% ± 1.4
n_{wells}, N_{plates}	n= 12, N= 4	n= 12, N= 4	n= 12, N= 4

b Lab II	Isoproterenol concentration (nM) (t= 10 min)		
	3	10	30
Spike amplitude	+36 ± 7.3	+24 ± 5.8	+36 ± 8.9
Beat rate	+23 ± 4.8	+28 ± 6.0	+43 ± 6.2
FPD	-24 ± 2.4	-34 ± 0.8	-55 ± 2.9
FPD_c	-19 ± 1.4	-28 ± 0.9	-48 ± 2.7
Wells active	100% ± 0	100% ± 0	98% ± 2.4
Electrodes active	12/12	6/12^	6/12^^
n_{wells}, N_{plates}	n= 12, N= 3	n= 12, N= 3	n= 12, N= 3

^: 6/12 quiescence; ^^: 6/12 quiescence.

Supplementary Table VI. hiPSC-derived cardiomyocyte function: concentration-effect data of cocaine. The mean percentage change ± SEM between baseline and cocaine exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. X: concentrations not measured, -B: no beats/quiescence, and -P: flat repolarization peak.

	Cocaine concentration (μM) (t= 30 min)							
	0.01	**0.1**	**1**	**10**	**30**	**100**	**300**	**1000**
Spike amplitude	+8.4 ± 8.7	+3.4 ± 3.1	-9.9 ± 6.1	-51 ± 9.1	-78 ± 3.5	-B	X	X
Beat rate	-6.8 ± 7.5	-0.6 ± 4.7	+8.1 ± 8.8	-44 ± 12	-85 ± 12	-B	X	X
FPD	+5.3 ± 5.1	+2.3 ± 4.5	+13 ± 4.9	+149 ± 43	-P	-B	X	X
FPD_c	+3.8 ± 3.0	+3.0 ± 3.3	+16 ± 4.5	+110 ± 27	-P	-B	X	X
Wells active	5/5	5/5	5/5	5/5	3/6^	0/5^^	X	x
Electrodes active	100% ± 0	100% ± 0	93% ± 4.3	83% ± 4.6	54% ± 2.9	-	X	X
n_{wells}, N_{plates}	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	n= 6, N= 3	n= 5, N= 3	X	X

^: 3/6 flat repolarization peak, 3/6 quiescence; ^^: 5/5 quiescence.

Supplementary Table VII. hiPSC-derived cardiomyocyte function: concentration-effect data of MDMA. The mean percentage change ± SEM between baseline and MDMA exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. X: concentrations not measured, -B: no beats/quiescence, and -P: flat repolarization peak.

	MDMA concentration (μM) (t= 30 min)							
	0.01	**0.1**	**1**	**10**	**30**	**100**	**300**	**1000**
Spike amplitude	-6.8 ± 3.8	+20 ± 4.8	-4.1 ± 3.5	-12 ± 8.2	X	-56 ± 12	-74 ± 6.6	-B
Beat rate	-0.5 ± 9.5	-0.5 ± 10	+1.8 ± 8.8	+3.9 ± 3.5	X	-5.9 ± 17	-82 ± 4.1	-B
FPD	-0.4 ± 5.0	+4.2 ± 1.7	+4.5 ± 0.9	+3.3 ± 2.6	X	+50 ± 10	-P	-B
FPD_c	-0.3 ± 3.4	+4.8 ± 1.6	+6.0 ± 2.0	+5.6 ± 2.7	X	+47 ± 1.6	-P	-B
Wells active	5/5	5/5	5/5	5/5	X	5/5	4/7^	0/6^^
Electrodes active	94% ± 4.2	91% ± 8.6	95% ± 3.3	100% ± 0	X	79% ± 5.8	47% ± 9.0	-
n_{wells}, N_{plates}	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	X	n= 5, N= 3	n= 7, N= 3	n= 6, N= 3

^: 4/7 flat repolarization peak, 3/7 quiescence; ^^: 6/6 quiescence.

Supplementary Table VIII. hiPSC-derived cardiomyocyte function: concentration-effect data of amphetamine. The mean percentage change ± SEM between baseline and amphetamine exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. X: concentrations not measured, and -B: no beats/quiescence.

	Amphetamine concentration (μM) (t= 30 min)							
	0.01	**0.1**	**1**	**10**	**30**	**100**	**300**	**1000**
Spike amplitude	-0.8 ± 5.1	-0.2 ± 5.4	-5.5 ± 4.8	-3.4 ± 8.3	X	-34 ± 9.5	-80 ± 5.9	-B
Beat rate	-0.6 ± 6.9	+3.3 ± 4.7	-1.4 ± 4.8	+20 ± 18	X	+0.4 ± 6.5	-28 ± 14	-B
FPD	+1.7 ± 1.6	-0.8 ± 2.4	+0.1 ± 1.7	+5.7 ± 2.5	X	+14 ± 1.9	+54 ± 17	-B
FPD_c	+2.3 ± 0.9	+0.9± 2.2	+0.6 ± 1.1	+12 ± 2.8	X	+16 ± 2.6	+43 ± 6.6	-B
Wells active	5/5	5/5	5/5	5/5	X	5/5	5/6^	0/8^^
Electrodes active	88% ± 7.3	100% ± 0	100% ± 0	97% ± 2.9	X	93% ± 4.5	66% ± 10	-
n_{wells}, N_{plates}	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	X	n= 5, N= 3	n= 6, N= 3	n= 8, N= 3

^: 1/6 flat repolarization peak, 1/6 quiescence; ^^: 8/8 quiescence.

Supplementary Table IX. hiPSC-derived cardiomyocyte function: concentration-effect data of 4-FA. The mean percentage change ± SEM between baseline and 4-FA exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. X: concentrations not measured, and -B: no beats/quiescence.

	4-FA concentration (μM) (t= 30 min)							
	0.01	**0.1**	**1**	**10**	**30**	**100**	**300**	**1000**
Spike amplitude	-7.9 ± 5.2	-6.7 ± 5.7	-13 ± 4.1	-4.0 ± 5.8	X	-57 ± 8.1	-97	-B
Beat rate	-7.8 ± 4.6	-1.5 ± 5.4	+7.0 ± 7.0	+12 ± 8.6	X	+5.4 ± 6.5	-65	-B
FPD	+3.0 ± 2.3	-1.4 ± 1.5	+0.9 ± 1.4	+7.2 ± 2.3	X	+16 ± 3.5	+526	-B
FPD_c	+1.5 ± 1.8	-1.0 ± 2.8	+4.0 ± 2.8	+12 ± 4.4	X	+19 ± 2.7	+303	-B
Wells active	5/5	5/5	5/5	5/5	X	5/5	1/7^	0/6^^
Electrodes active	100% ± 0	100% ± 0	98% ± 2.5	97% ± 2.9	X	70% ± 15	75%	-
n_{wells}, N_{plates}	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	n= 5, N= 3	X	n= 5, N= 3	n= 7, N=3	n= 6, N=3

^: 6/7 quiescence; ^^: 6/6 quiescence.

Supplementary Table X. hiPSC-derived cardiomyocyte function: concentration-effect data of MDPV. The mean percentage change ± SEM between baseline and MDPV exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. -B: no beats/quiescence, and -P: flat repolarization peak.

	MDPV concentration (µM) (t= 30 min)							
	0.01	**0.1**	**1**	**10**	**30**	**100**	**300**	**1000**
Spike amplitude	-11 ± 5.5	-1.3 ± 9.4	-9.4 ± 3.8	-36 ± 6.6	-68 ± 4.1	-96 ± 2.4	-B	-B
Beat rate	-1.7 ± 2.5	+5.1 ± 2.9	-2.8 ± 1.7	-12 ± 14	-45 ± 7.3	-63 ± 3.3	-B	-B
FPD	-4.3 ± 1.1	-3.6 ± 2.9	+3.1 ± 2.1	+91 ± 40	+182 ± 95	-P	-B	-B
FPD_c	-4.2 ± 1.1	-1.3 ± 2.7	+3.3 ± 1.7	+68 ± 26	+130 ± 52	-P	-B	-B
Wells active	7/7	7/7	7/7	7/7^	7/7	3/7^^	0/7^^^	0/7^^^^
Electrodes active	98% ± 1.8	98% ± 2.4	100% ± 0	96% ± 2.9	81% ± 9.8	18% ± 3.7	-	-
n_{wells}, N_{plates}	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3

^: 1/7 flat repolarization peak; ^^: 3/7 flat repolarization peak, 4/7 quiescence; ^^^: 7/7 quiescence; ^^^^: 7/7 quiescence.

Supplementary Table XI. hiPSC-derived cardiomyocyte function: concentration-effect data of α-PVP. The mean percentage change ± SEM between baseline and α-PVP exposure measurements, corrected for vehicle control, is listed for several parameters: spike amplitude, beat rate, the (corrected) field potential duration ($FPD_{(c)}$), the number of active wells, and the percentage of active electrodes in the remaining active wells (see methods 2.4 for details). Effects (decrease (-) in purple, increase (+) in orange) were considered relevant if significantly different from control ($p<0.05$) and larger than the threshold based on the variation of the vehicle control. -B: no beats/quiescence.

	α-PVP concentration (µM) (t= 30 min)							
	0.01	**0.1**	**1**	**10**	**30**	**100**	**300**	**1000**
Spike amplitude	-5.1 ± 5.8	-10 ± 3.7	-3.2 ± 5.5	-16 ± 4.8	-35 ± 7.9	-74 ± 6.2	-101 ± 2.5	-B
Beat rate	+0.5 ± 3.7	+2.2 ± 3.0	+3.2 ± 4.1	+2.9 ± 0.9	+3.7 ± 2.2	-14 ± 13	-51 ± 2.5	-B
FPD	-4.5 ± 2.3	-4.0 ± 1.8	-4.7 ± 2.1	-4.4 ± 1.3	+0.2 ± 3.9	+54 ± 24	+158 ± 40	-B
FPD_c	-5.8 ± 0.9	-2.5 ± 1.5	-2.3 ± 1.6	-3.1 ± 1.0	+1.6 ± 3.6	+33 ± 15	+84 ± 21	-B
Wells active	7/7	7/7	7/7	7/7	7/7	7/7	3/7^	0/7^^
Electrodes active	98% ± 1.8	100%± 0	100% ± 0	98% ± 1.8	95% ± 2.5	56% ± 7.3	19% ± 4.8	-
n_{wells}, N_{plates}	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3	n= 7, N= 3

^: 1/7 flat repolarization peak, 4/7 quiescence; ^^: 7/7 quiescence.