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Research Article

Is the time right for *in vitro* neurotoxicity testing using human iPSC-derived neurons?

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Summary
Current neurotoxicity testing heavily relies on expensive, time consuming and ethically debated *in vivo* animal experiments that are unsuitable for screening large number of chemicals. Consequently, there is a clear need for (high-throughput) *in vitro* test strategies, preferably using human cells as this increases relevance and eliminates the need for interspecies translation. However, human stem cell-derived neurons used to date are not well characterised, require prolonged differentiation and are potentially subject to batch-to-batch variation, ethical concerns and country-specific legislations. Recently, a number of human induced pluripotent stem cell (iPSC)-derived neurons became commercially available that may circumvent these concerns.
We therefore used immunofluorescent stainings to demonstrate that human iPSC-derived neurons from various suppliers form mixed neuronal cultures, consisting of different types of (excitatory and inhibitory) neurons. Using multi-well microelectrode array (mwMEA) recordings, we demonstrate that these human iPSC-derived cultures develop spontaneous neuronal activity over time, which can be modulated by different physiological, toxicological and pharmacological compounds. Additional single cell calcium imaging illustrates the presence of functional GABA, glutamate, and acetylcholine receptors as well as voltage-gated calcium channels.
While human iPSC-derived neuronal cultures appear not yet suitable to fully replace the rat primary cortical model, our data indicate that these rapidly differentiating, commercially available human iPSC-derived neuronal cultures are already suitable for *in vitro* prioritisation and effect screening studies. Further characterisation and toxicological validation is now required to facilitate acceptance and large-scale implementation of these animal-free, physiologically-relevant human iPSC-based modelsfor future neurotoxicity testing.

Keywords: *In vitro* neurotoxicity screening, multi-electrode array (MEA), human induced pluripotent stem cell-derived neurons, mixed neuronal cultures, alternatives to animal testing

1 Introduction[1]

Current neurotoxicity testing heavily relies on animal experiments. These *in vivo* experiments are not only ethically debated, but are also expensive and time consuming and therefore unsuitable for screening large numbers of chemicals (Bal-Price et al., 2008). As a result, there is a clear need for the development of innovative (high-throughput) *in vitro* test strategies. Such *in vitro* strategies not only reduce animal use, but also increase mechanistic insight in the modes of action of the tested chemicals (Bal-Price et al., 2008, 2010; Llorens et al., 2012; van Thriel et al., 2012). However, *in vitro* neurotoxicity testing receives considerable criticism and scepticism, in particular from a regulatory perspective, as it has been suggested that *in vitro* models are too simplified to mimic the complexity of the *in vivo* brain and are not predictive enough. However, these concerns seem unjustified if the *in vitro* models used and endpoints assessed meet some defined basic requirements (Westerink, 2013).

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[1]**Abbreviations**
β(III)tubulin, class III β-tubulin; DAPI, 4,6-diamidino-2-phenylindole; DIV, days *in vitro*; GFAP, glial fibrillary acidic protein; iPSC, induced pluripotent stem cell; MEA, microelectrode array; MSR, mean spike rate; mwMEA, multi-well microelectrode array; PND, post-natal day; RFU, relative fluorescence unit; rt, room temperature; TR, treatment ratio; vGAT, vesicular GABA transporter; vGluT, vesicular glutamate transporter.

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In vitro screening models should represent the *in vivo* situation as closely as possible. For neurotoxicity testing this basically translates into an *in vitro* model that forms functional neuronal networks consisting of a mixed population of multiple types of (excitatory and inhibitory) neurons as well as supportive cells (e.g., astrocytes) to capture the complexity of the brain. The cellular morphology and heterogeneity of the formed neuronal networks can be assessed using immunofluorescent staining of specific proteins to identify the presence of astrocytes and (excitatory and inhibitory) neurons. While subsequent electrophysiological assessment of neuronal function is ideally suited to investigate chemically-induced changes in neuronal function and transmission (de Groot et al., 2013), it is often too endpoint-specific, requires training and expertise, and lacks the required throughput.

The recent introduction of microelectrode array (MEA) recordings bypasses these limitations. MEAs consist of a cell culture surface with an integrated array of microelectrodes that allows for the simultaneous and non-invasive recordings of (extracellular) local field potentials at different locations in an *in vitro* neuronal network at millisecond time scale (for review, see Johnstone et al., 2010). Neuronal networks grown on MEAs possess many characteristics of neurons *in vivo*, including (the development of) spontaneous activity (Robinette et al., 2011) and responsiveness to neurotransmitters and pharmacological agents (Gross et al., 1997; for review, see Johnstone et al., 2010; de Groot et al., 2013). As such, neuronal networks on MEAs have been proposed as an *in vitro* neurotoxicity screening method that shows consistent reproducibility and reliability across different laboratories (Novellino et al., 2011) as well as high sensitivity and specificity (McConnell et al., 2012; Valdivia et al., 2014; Nicolas et al., 2014).

While many different *in vitro* models can be cultured on MEAs, primary rat cortical cultures are the current standard (Hogberg et al., 2011; McConnell et al., 2012; Valdivia et al., 2014). These primary cultures are well characterised, widely accepted, easily cultured and recapitulate many aspects of nervous system function (for review, see Johnstone et al., 2010, de Groot et al., 2013). However, as also outlined in NC3Rs'Neuratect Crack-it Challenge (www.NC3Rs.org.uk), the use of human neurons for *in vitro* neurotoxicity testing is preferred. Nevertheless, the use of human neurons for MEA recordings has been limited (Heikkilä et al., 2009;Ylä-Outinen et al., 2010;Kapucu et al., 2012), while there is no indication that human-derived neuronal networks will not exhibit the same properties as primary rat cortical cultures.

Importantly, interspecies translation is no longer necessary ifhuman-derived (stem) cells are used to assess chemically-induced effects on proliferation, migration and differentiation as well as other endpoints relevant for human (developmental) neurotoxicity (for review, see de Groot et al., 2013). However, human stem cell-derived neurons used to date are less well characterised than cortical neuronsand potentially subject to batch-to-batch variation, which could hamper data interpretation. Moreover, these human stem cell-derived neurons require extensive (>3 weeks) and costly differentiation on the MEA, making them less amenable to screening large numbers of chemicals in an efficient and timely manner. Finally, human stem cell-derived neurons may not be readily available for use in all laboratories as a result of restrictions due to ethical concerns and country-specific legislations.In recent years, a considerable number of human induced pluripotent stem cell (iPSC)-derived neurons became commercially available that may circumvent these difficulties. The potential of these human iPSC-derived neurons for *in vitro* neurotoxicity testing is however not yet firmly established.

We therefore purchased human iPSC-derived neurons from various commercial suppliers to demonstrate their mixed neuronal nature using immunofluorescent staining. Importantly, by characterising both the functional presence of neurotransmitter receptors and ion channels using single cell calcium imaging as well as the development and modulation of spontaneous neuronal network activity using mwMEA recordings we demonstrate their potential for future high-throughput neurotoxicity testing. Upon further characterisation and toxicological validation, the use of such physiologically-relevant human *in vitro*iPSC-based models can facilitate predictions of toxicological profiles and improve human risk assessment, while reducing or even replacing animal experimentsfor neurotoxicity testing.

2 Materials and Methods

2.1 Chemicals

Neurobasal®-A Medium, DMEM with high glucose, L-glutamine, fetal bovine serum (FBS), N2 supplement, KnockOut Serum Replacement,Natural Mouse Laminin, penicillin-streptomycin (10000 U/mL-10000 µg/mL),GlutaMAX,Geltrex,B27 supplement (without vitamin A)and Fura-2 AM were purchased from Life Technologies (Bleiswijk, The Netherlands). iCell Neurons Maintenance Medium (NRM-100-121-001) andiCell Neurons Medium Supplement (NRM-100-031-001) were purchased from Cellular Dynamics international (Madison, WI, USA). Pre-Coat Solution (GSM-9450), NeuralQ™ Basal Medium (GSM-9420) and GS21™ Neural Supplement (GSM-3100) were purchased from MTI-GlobalStem (Gaithersburg, MD, USA). GlutaGRO was purchased from Corning Incorporated (Corning, NY, USA). DOPA.4U®Thawing Medium, DOPA.4U®Culture Medium and DA-supplement were purchased from Axiogenesis (Cologne, Germany). DL-Amphetamine was obtained from SpruytHillen (IJsselstein, The Netherlands).Poly-L-Ornithine (PLO), 50% polyethyleneimine-coated (PEI) solution, laminin, Sodium Borate, Boric Acid and all other chemicals (unless otherwise described) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Saline solution for Ca^{2+} imaging experiments (containing (in mM) 125 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose, and 36.5 sucrose) and high-potassium saline solution (containing (in mM) 5.5 NaCl, 100 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose and 36.5 sucrose) were prepared with deionized water (Milli-Q; resistivity; 10 MΩ·cm). For both saline solutions, pH was set at 7.3. Saline solutions containing gamma-aminobutyric acid (GABA 100 µM), acetylcholine chloride (ACh100 µM) or sodium L-glutamic acid (glutamate 100 µM) were prepared in separate aliquots for every experimental day and kept at -20°C until use.

2.2 Cell culture

Cells were cultured at 37°C in a humidified 5% CO_2 atmosphere according to supplier instructions. Briefly, ***iCell® Neurons*** (NRC-100-010-001, Cellular Dynamics international, Madison, WI, USA) were seeded in Complete iCell Neurons Maintenance Medium (with 2% iCell Neurons Medium Supplement) supplemented with laminin (10 µg/mL). For immunofluorescent stainings, iCell® Neurons were seeded at a density of 100.000 cells/chamber on Poly-L-Ornithine-coated ([PLO] 0.01%) 8-chamber coverslips (Ibidi GmbH, Planegg, Germany). For MEA experiments, 70.000 cells/well were seeded as a 4 µL droplet of cell suspension directly over the electrode field of each well of a 48-well MEA plate (Axion Biosystems Inc., Atlanta, USA) coated with 0.1% polyethyleneimine ([PEI] solution diluted in borate buffer [24 mM Sodium Borate/50 mM Boric Acid in Milli-Q adjusted to pH 8.4]). The droplet of cell suspension was allowed to adhere to the electrode field for 40 min, after which 300 µL of CompleteiCell Maintenance Medium supplemented with laminin was added to each well. For Ca^{2+} imaging experiments, cells were seeded at a density of 175.000 cells/dish as a 300 µL droplet on the glass of PEI-coated glass-bottom dishes (MatTek, Ashland, MA). The droplet of cell suspension was allowed to adhere to the glass for 40 min after which 1.5 mL of CompleteiCell Maintenance Medium supplemented with laminin was added. After one to three days *in vitro* (DIV1 to 3), 50% of the medium in the coverslips was replaced by Complete Maintenance Medium, and 100% of the medium in the mwMEA plates and glass-bottom dishes was replaced by Neurobasal®-A (NBA) medium supplemented with 10% KnockOut Serum Replacement, 100 U/mL penicillin and 100 µg/mL streptomycin. Every 3-4 days, 50% of the medium was refreshed: in coverslips by Complete Maintenance Medium and in the mwMEA plates and glass-bottom dishes by supplemented NBA medium. For ***iCell® Neurons/CDI iCell® Astrocytes co-cultures***, iCell® Neurons were seeded as described above. For immunofluorescent stainings, iCell® neurons were seeded at a density of 66.000 cells/chamber in 150 µLComplete Maintenance Medium supplemented with laminin on PLO-coated coverslips. iCell neurons were allowed to adhere for 40 min, after which iCell® Astrocytes (ASC-100-020-001-PT, Cellular Dynamics international, Madison, USA), thawed in DMEM with high glucose + 10% fetal bovine serum (FBS) and 1% N2 supplement, were seeded at a density of 22.000 cells/chamberin 150 µLlaminin-supplemented Complete Maintenance Medium in each chamber on the coverslip. Co-cultureswere refreshed as described above for the CDI iCell Neurons. ***HIP neurons*** (GSC-4312, MTI-GlobalStem, Gaithersburg, MD, USA) were seeded in Neuronal Maintenance Medium (NeuralQ™ Basal Medium supplemented with 2% GS21™ Neural Supplement and 2 mMGlutaGRO). For immunofluorescent stainings100.000 cells/chamber were seeded on coverslips and for MEA measurements, 90.000 cells/well were seeded as a 4 µL droplet directly over the electrode field of each MEA well. Cells were allowed to attach for 20 min at room temperature (rt) and a subsequent 30 min in a humidified incubator at 37°C and 5% CO_2 after which 300 µL Neuronal Maintenance Medium was added to each well. OnDIV1 and every 3-4 days thereafter, 50% of the Neuronal Maintenance Medium was replaced by fresh Neuronal Maintenance Medium. All culture materials were coated with 1x GS Pre-Coat solution and Natural Mouse Laminin (15 µg/mL). ***DOPA.4U® neurons*** (Axiogenesis, Cologne, Germany) were thawed in DOPA.4U®Thawing Medium supplemented with laminin (10 µg/mL) and seeded in DOPA.4U®Culture Medium (supplemented with 2.5% DA-supplement and laminin (10 µg/mL)). For immunofluorescent stainings, 52.000 cells/chamber were seeded on coverslips. For MEA experiments, 40.000 cells/well were seeded as a 5 µL droplet directly over to the electrode field of the MEA wells. Cells were allowed to adhere to the electrode field for 1 hour, after which 300 µL of laminin-supplemented DOPA.4U®Culture Medium was added to each well. Medium was refreshed every 2-3 days with DOPA.4U®Culture Medium. All culture materials were coated with Geltrex. For ***DOPA.4U® neurons/iCell® Astrocytes co-cultures***, DOPA.4U® neurons were seeded as described above. For immunofluorescent stainings, 48.000 cells/chamberwere seeded on coverslips in 150 µLDOPA.4U®Culture Medium supplemented with laminin. Cells were allowed to adhere to the coverslips for 1 hour, after which 150 µL of iCell Astrocytes, thawed in DMEM with high glucose + 10% FBS and 1% N2 supplement, were added in a density of 20.000 cells/chamberin laminin-supplemented DOPA.4U®Culture Medium. Medium was refreshed every 2-3 days with DOPA.4U®Culture Medium. All culture materials were coated with Geltrex. ***Primary rat cortical cultures*** were isolated from post-natal day (PND)1Wistar rat pups in accordance with the Dutch law and approved by the Ethical Committee for Animal Experimentation of Utrecht University. Animals were treated humanely and efforts for alleviation of suffering were taken. Culture preparation was done as described previously (Nicolas et al., 2014; de Groot et al., 2016). Briefly, PND1 rat pups were decapitated and cortices were rapidly isolated on ice and kept in dissection medium (Neurobasal-A supplemented with sucrose [25 g/l], L-glutamine [450 µM], glutamate [30 µM], penicillin/streptomycin [1%], and FBS [10%], pH 7.4) during the entire isolation. Cells were seeded in dissection medium on poly-L-lysine (50 µg/mL) coated culture materials following which cells were kept at 37°C in a 5% CO_2 atmosphere. For MEA experiments, 100.000 cells/well were seeded as a 50 µL droplet directly over the electrode field of the MEA wells. Cells were allowed to adhere for 2 hours, after which 450 µL of dissection medium was added to each well. At DIV1, 90% of the dissection medium was replaced with glutamate medium (Neurobasal-A supplemented with sucrose [25 g/L], L-glutamine [450 µM], glutamate [30 µM], penicillin/streptomycin [1%], and B-27 supplement [2%], pH 7.4). At DIV4 and DIV11, 90% of the culture medium was replaced with FBS medium (Neurobasal-A supplemented with sucrose [25 g/L], L-glutamine [450 µM], penicillin/streptomycin [1%], and FBS [10%], pH 7.4).

2.3 Immunocytochemistry

Different human iPSC-derived neurons were fixed on DIV8-14 with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 15 min at room temperature (rt). Subsequently, coverslips were quenched for PFA, permeabilised, and incubated with blocking buffer (2% bovine serum albumin and 0.1% saponin in PBS) containing 20 mM NH_4Cl for 20 min at rt. Each of the subsequent wash and incubation steps was performed in blocking buffer. Next, coverslips were incubated overnight at 4ºC with rabbit anti-βIII tubulin (ab18207, Abcam, Cambridge, United Kingdom) at a final dilution of 1:500, goat anti-GFAP (ab53554, Abcam, Cambridge, United Kingdom) at a final dilution of 1:100, mouse anti-vGAT(131 001, Synaptic Systems, Göttingen, Germany) at a final dilution of 1:1000, and/or rabbit anti-vGluT1 (ab104898, Abcam, Cambridge, United Kingdom) at a final concentration of 1:1500. Subsequently, coverslips were washed 3 times with blocking buffer and

incubated with donkey anti-rabbit Alexa 488, donkey anti-goat 594, donkey anti-mouse Alexa 488, and/or donkey anti-rabbit 594 (Life Technologies, Bleiswijk, The Netherlands) at a final dilution of 1:100 for 30 min at rt in the dark. Nuclear staining was performed by incubating the coverslips with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies, Bleiswjik, The Netherlands) at a concentration of 200 nM for 2-3 min at rt in the dark. The washing procedure was repeated and coverslips were sealed with FluorSave (Calbiochem, San Diego, California). Immunostained coverslips were visualized using a Leica SPEII Confocal microscope (Leica DMI4000 equipped with TCS SPE-II) using a ×20 oil immersion objective (N.A. 1.4-0.7) and images were captured as *.tif files using Leica Application Suite Advanced Fluorescence software (LAS AF version 2.6.0; Leica Microsystems GmbH, Wetzlar, Germany).

2.4 MEA measurements

Different human iPSC-derived neurons were cultured on 48-well MEA plates, with each well containing an electrode array of 16 nanotextured gold microelectrodes (~40–50 µm diameter; 350 µmcenter-to-center spacing) with 4 integrated ground electrodes, yielding a total of 768 channels that can be recorded simultaneously (see Figure 1 for illustration). Spontaneous electrical activity is recorded as described previously (Nicolas et al., 2014; de Groot et al. 2014; de Groot et al. 2016). Briefly, signals were recorded at various culture durations (DIV4-9) using a Maestro 768-channel amplifier with integrated heating system and temperature controller and a data acquisition interface (Axion Biosystems Inc.). Axion's Integrated Studio (AxIS 2.0.2.11) was used to manage data acquisition. Prior to the 30 min recording of spontaneous activity, MEA plates were allowed to equilibrate in the Maestro for ~5 min. To obtain raw data files, channels were sampled simultaneously at a constant temperature of 37°C with a gain of 1200× and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200–5000 Hz).

To determine changes in spontaneous neuronal activity of iCell neurons(without added astrocytes), activity was recorded for 30 min at DIV7-8. Immediately following this baseline recording, cells were exposed (33 µL/well, i.e. 1:10 dilution) to test compounds or appropriate (solvent) controls and activity was recorded for another 30 min. Stock (10x) solutions of glutamate, GABA, receptor (ant)agonists, endosulfan, and DL-amphetamine were diluted in cell culture medium to obtain the desired concentrations.

2.5 Intracellular calcium imaging

For further characterisation ofiCell neurons(without added astrocytes), basal and stimulation-evoked changes in $[Ca^{2+}]_i$ were measured at DIV4-8 using the Ca^{2+}-sensitive ratio fluorescent dye Fura-2 AM as described previously (de Groot et al., 2014; de Groot et al., 2016). In short, cells were loaded with 5 µM Fura-2AM for 25 min at rt. Following 20 min to allow for de-esterification of the dye, Fura-2-loaded cells were placed under the microscope under continuous superfusion with saline. Following a 5 min baseline measurement, cells were stimulated for 21 s with high-K^+ saline (100 mM K^+), 100 µM GABA, 100 µM glutamate, or 100 µMACh using an automated continuous superfusion system (AutoMate Scientific Inc., Berkeley, California). Hereafter, superfusion solution was switched back to saline allowing the cells to recover for 10 min. Each dish of cells received only one stimulation to prevent potential effects of foregoing stimuli.

2.6 Data analysis and statistics

In order to calculate the percentage of astrocytes present in the different cultures the average number of astrocytes and total number of cells were counted (independently by two different researchers) from *n*images taken from *N* different cultures with Leica Application Suite Advanced Fluorescence software. Data are presented as mean ±SEM.

To analyse (modulation of) spontaneous neuronal activity, raw data files were re-recorded to obtain Alpha Map files for further data analysis in NeuroExplorer. During the re-recording, spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a variable threshold spike detector set at 6×(human iPSCs) or 7× (primary cortical culture) standard deviation (SD) of the internal noise level (rms) on each electrode. Next, spike count files were loaded into NeuroExplorer 5.007 software (Nex Technologies, Madison, Wisconsin) to determine the average mean spike rate (MSR; spikes/s) of all active electrodes (>0.01 spikes/s) per active well (≥1 active electrode).

Custom-made excel macros were used to determine the development of spontaneous activity (MSR) over time and to determine the effect of test compounds on spontaneous activity. For the latter, a treatment ratio (TR) was established by: $(MSR_{exposure}/MSR_{baseline}) \times 100\%$. Next, TRs of exposures were normalized to the appropriate medium (glutamate, GABA, amphetamine) or DMSO (endosulfan) controls. Outliers in control and effect data (defined as not within average ± 2 × SD) were removed (<5%). All data are presented as mean ± SEM from the number of electrodes (*n*) or wells (*N*) indicated. A t-test was performed to determine significant changes in MSR in comparison to the control. Differences were considered significant if $p< 0.05$.

For calcium imaging experiments, changes in F_{340}/F_{380}ratios reflecting changes in $[Ca^{2+}]_i$ were analysed using custom-made MS-Excel macros. Recordings were normalized to baseline, and changes in $[Ca^{2+}]_i$ are expressed as relative fluorescence units (RFU).Per stimulus condition, the percentage of active cells (defined as change in $[Ca^{2+}]_i$>1.2 RFU upon stimulation) was calculated and used for subsequent analysis of the amplitude of $[Ca^{2+}]_i$. Outliers (defined as cells with amplitude$[Ca^{2+}]_i$ not within average ± 2 × SD) were removed from the data set (<5%). All data are presented as mean ± SEM from the number of cells (*n*) or dishes (*N*) indicated.

3Results

3.1 Immunofluorescent staining of human iPSC-derived neurons

As an initial characterisation and to demonstrate their mixed neuronal nature, human iPSC-derived neurons were cultured for up to 1-2 weeks and β(III)tubulin and glial fibrillary acidic protein (GFAP) antibodies were used to identify the presence of respectively neurons and astrocytes. HIP neurons (Figure 2, upper left) show a mixed phenotype with both neurons and astrocytes (21 ± 7% astrocytes; *n*=5 images originating from 3 independent cultures). On the other hand, iCell neurons and DOPA.4U neurons have a more pure neuronal phenotype with very few astrocytes (not shown). Our initial experiments demonstrate that the heterogeneity of these cells can easily be increased by co-culturing the cells with pure astrocytes (Figure 2, lower left (DOPA.4U, 14 ± 3% astrocytes; *n*=3 images originating from a single culture) and upper right (iCell Neurons, 20 ± 2% astrocytes;*n* = 4 images originating from a single culture)). However, these co-cultures contain fewer astrocytes than the primary rat cortical culture (46 ± 4%;*n* = 8 images originating from 3 independent cultures).

To discriminate between different subtypes of neurons, we performed additional stainings for vesicular transporters of glutamate (vGluT) and GABA (vGAT). HIP neurons (not shown), iCell neurons (not shown) and iCell neurons co-cultured with pure astrocytes (Figure 2, lower right) all show clear expression of vGluT and vGAT, indicating the coexistence of respectively excitatory and inhibitory neuronal contacts.

These combined data indicate that our different human iPSC-derived (co-)cultures consist of excitatory glutamatergic neurons, inhibitory GABAergic neurons and (added) supporting astrocytes. The derived neuronal networks thus represent the cellular heterogeneity as observed in the *in vivo* brain and could be amenable to neurotoxicity testing.

3.2 Spontaneous electrical activity of human iPSC-derived neurons

To further test the applicability of these human iPSC-derived neurons for neurotoxicity testing, cells were cultured(without addition of astrocytes) on mwMEAs for up to9 days to assess the development of spontaneous neuronal network activity over time. Electrical activity at DIV7-8 is depicted in spike raster plots,activity heat maps and individual spike traces (Figure 3). As can be seen in the rasterplots and heat maps, neuronal activity differs between different cell models, with the iCell neurons and rat primary cortex cultures being the most active at DIV7-8. As also shown in table 1,HIP neurons develop spontaneous neuronal activity following 4-6 days in culture, which slightly increases following 7-9 days in culture. Similarly, DOPA.4U neurons develop spontaneous neuronal activity that steadily increases over time. While the MSR of DOPA.4U neurons is clearly increased compared to HIP neurons, the % active wells and % active electrodes is comparable.

Although MSR of HIP neurons and DOPA.4U neurons may further increase with prolonged differentiation in culture, such prolonged differentiation protocols make the cells less amenable to efficient and timely neurotoxicity screening. On the other hand, iCell neurons appear already maximally active following 4-6 days in culture, with a high MSR, % active wells and % active electrodes that slightly decreases with prolonged culture.

Primary rat cortical cultures, which are the current standard model for MEA recordings, are not yet active following 4-6 days in culture.Following 7-9 days of culture, activity of primary rat cortical cultures is comparable to the activity of iCell neurons at DIV4-6 (table 1), although it should be noted that the electrical activity of primary rat cortical cultures will increase with prolonged culture duration (see e.g. de Groot et al., 2016; Dingemans et al., unpublished).

3.3 Modulation of electrical activity of iCell neurons

While all tested cell cultures develop spontaneous activity, the rapid development of activity of iCell neurons can clearly be an advantage for neurotoxicity screening. Consequently, we used iCell neurons (without addition of astrocytes) for an initial brief functional screening using physiological, pharmacological and toxicological stimuli (Figure 4A-B). To verify the presence of glutamate and GABA receptors in iCell neurons, cells were exposed to different concentrations of glutamate (0.3 – 30 μM) and GABA (1 – 3 μM). Results demonstrate that spontaneous neuronal activity is concentration-dependently inhibited by high concentrations of glutamate and GABA following acute exposure (Figure 4A). Moreover, the neurotoxic insecticide endosulfan, which is a $GABA_A$ receptor antagonist, clearly increases neuronal activity (Figure 4B), comparable with primary rat cortical neurons challenged with endosulfan(Dingemans et al., unpublished) or the $GABA_A$ receptor antagonist lindane (Wallace et al., 2015). Finally, when iCellneurons were challenged with the widely used drug of abuse amphetamine, the MSR shows a concentration-dependent decrease (Figure 4B) as also observed for primary rat cortical neurons (Hondebrink et al., unpublished).

3.4 Functional characterisation of receptors and ion channels in iCell neurons

To further characteriseiCell neurons with respect to the presence of receptors and ion channels, $[Ca^{2+}]_i$ was measured using single-cell fluorescence microscopy. iCell neurons display a robust transient increase in $[Ca^{2+}]_i$ upon depolarisation with K^+-containing saline (Figure 5A), demonstrating the presence of functional voltage-gated calcium channels. Also, stimulation with glutamate (100 μM), GABA (100 μM) and ACh (100 μM) results in an transient increase in $[Ca^{2+}]_i$, indicative for the presence of Ca^{2+} permeable (ionotropic) glutamate receptors, GABA receptors and nicotinic ACh receptors (nACh-R), respectively (Figure 5A).In addition to stimulation-evoked transient increases in $[Ca^{2+}]_i$,iCell neurons exhibited spontaneous activity and occasionally synchronicity could be observed (Figure 5B).

4 Discussion

Reliable detection of compound-induced neurotoxicity is challenging due to the high degree of integration of neuro-cellular processes in the (central) nervous system (CNS). Consequently, neurotoxicity testing currently relies strongly on animal studies, which are mandatory for new chemicals and CNS drugs, but require hundreds of rodents and up to a million euro per test compound. Successful and cost-effective replacement of these *in vivo* tests will thus drastically impact animal use for regulatory requirements. The use of integrated endpoints such as neuronal network activity allows for assessment of

physiologically-relevant effects with considerable throughput and high sensitivity and specificity *in vitro* (McConnell et al., 2012; Valdivia et al., 2014; Nicolas et al., 2014), whereas direct effects on ion channels or neurotransmitter receptors could be assessed with targeted approaches such as single-cell calcium imaging. Importantly, the use of human cells prevents the need for interspecies extrapolation. While the costs of human iPSC-derived neurons are still high compared to primary cultures, the availability of human iPSC-derived neurons from particular brain areas and/or patients suffering from a particular neurological disorder opens the way to disease-specific neurotoxicity research (e.g., chemical-induced dopaminergic neurotoxicity or drugs screening using dopaminergic neurons like DOPA.4U).

Our immunocytochemical data (Figure 2) demonstrate that human iPSC-derived neurons from different suppliers efficiently capture the complexity and the high degree of integration of the brain in an *in vitro* system. iCell neurons, HIP neurons, DOPA.4U neurons and rat primary cortical cells all develop networks of inhibitory and excitatory neuronal cells when grown *in vitro*. The DOPA.4U and iCell neuron cultures had a relatively homogeneous neuronal nature with very little astrocytes present, whereas the HIP neuron culture had a more heterogeneous neuronal nature with a considerable fraction of astrocytes. The rat primary cortical cultures formed a heterogeneous network with presence of neurons and astrocytes, comparable with earlier findings (45% astrocytes; Görtz et al., 2004).

While it is long known that astrocytes can affect the vulnerability of primary cultures to toxic insults (e.g. Dugan et al., 1995), adding astrocytes to pure neuronal cultures may also alter neuronal development and enhance cell signalling (Clarke and Barres, 2013; Odawara et al., 2014). Moreover, addition of astrocytes may affect chemical sensitivity as it has been shown using a mixed culture of NTERA-2 (NT2) cells that astrocytes are more sensitive to e.g. lead chloride and aluminum nitrate than neurons (Laurenza et al. 2013). We therefore also created co-cultures of astrocytes with DOPA.4U neurons or iCell neurons. It is in theory possible to design cell cultures with different ratios of neurons vs. astrocytes, however, it remains to be determined in future research if and how different ratios of astrocytes affect development of neuronal activity and sensitivity of human iPSC-derived neurons to toxic insults.

Using mwMEA recordings, human neuronal cultures were shown to be functional (i.e., spontaneously active) and amenable to neurotoxicity screening, with the iCell neurons(without addition of astrocytes) being most activeat a short culture duration (DIV4-6; table 1, Figure 3). In accordance with previous studies (Robinette et al., 2011; Dingemans et al., unpublished), primary rat cortical cultures did not show any activity until DIV7. Around DIV7-9, primary cortical cultures and iCell neurons show a comparable degree of electrical activity.It should be noted though, that primary rat cortical cultures exhibit higher and stable levels of bursting and spiking following more prolonged culture (DIV10-14; Robinette et al., 2011; de Groot et al., 2016; Dingemans et al., unpublished) and that over time the degree of neuronal activity in DOPA.4U and HIP cultures may still develop to levels that equal or exceed those of iCell neurons at DIV4-6. While this should be taken into account in future studies, such studies may also need to focus on exploring the influence of different culture media on the speed and degree of neuronal development (see e.g. Bardy et al., 2015).Notably, our data on spontaneous network activity is derived from iCell and DOPA.4U neurons in the absence of (added) astrocytes. Future studiesmaytherefore also need to focus on co-cultures of human iPSC-derived neurons with astrocytes, as it has been shown that this can enhance firing frequency and bursting levels (Odawara et al., 2014).

Next to MSR, a number of additional parameters could theoretically be extracted from the MEA recordings, including the percentage of active wells or percentage of active electrodes. However, in comparison to primary rat cortical neurons, the percentage of active wells is somewhat lower in human iPSCs, especially for HIP neurons and DOPA.4U neurons (table 1). This low percentage of active electrodes effectively hampers analysis of bursting parameters and synchrony of electrical network activity. While rat primary cultures develop synchronised bursting (already visible at DIV7-8, Figure 3), our data indicate thatDOPA.4U neurons exertonly little bursting behaviour (Figure 3) and bursting was virtually absent in iCell neurons and HIP neurons. Bursting parameters may help identify different groups of chemicals (Alloisio et al., 2015; Mack et al., 2014), although it has previously been shown that adding bursting parameters does not result in an increased sensitivity to detect neurotoxic chemicals in MEA-based screening approaches (Defranchi et al., 2011; Shafer et al., 2008; Dingemans et al., unpublished) and the MSR alone provides sufficient information for a first screening (McConnell et al., 2012).Additionally, the increasing throughput of mwMEA systems, currently up to 96-wells with only 8 electrodes per well, will continue to hamper detailed analysis of network synchrony. Nevertheless, future experiments using human iPSCs should focus on identification of culture conditions that allow bursting behaviour and increase MSR as well as the percentage of active wells and the percentage of active electrodes. In this respect it should be noted that co-culturing human iPSC-derived neurons with astrocytes may enhance synaptic maturation and transmission (Clarke and Barres, 2013) as well as firing frequency and bursting behaviour (Odawara et al., 2014).

While DOPA.4U and HIP neurons may become more active over time, the rapid development of electrical activity and the higher spike rate of iCell neurons is a clear advantage for fast and efficient neurotoxicological screening. We therefore used the iCell neurons for an explorative functional screening using different physiological, pharmacological and toxicological stimuli.This initial assessment demonstrated that MEA data obtained with iCell neurons are largely in line with previous data from primary cultures (McConnell et al., 2012; Hogberg et al., 2011; Dingemans et al., unpublished; Hondebrink et al., unpublished) and human embryonic stem cell-derived neurons (Ylä-Outinen et al., 2010). Moreover, iCell neurons challenged with endosulfan or amphetamine (Figure 4), show changes in neuronal activity comparable with primary rat cortical neurons (Dingemans et al., unpublished; Hondebrink et al., unpublished), indicating not only the presence and functionality of different neurotransmitter receptors, but also the potential for chemical neurotoxicity screening.
The presence of functional neurotransmitter receptors (GABA, glutamate and acetylcholine receptors) and voltage-gated calcium channels early in culture was confirmed using single cell calcium imaging (Figure 5). These findings extend on data from Dage et al. (2014), who showed that iCell neurons respond with robust calcium transients in response to GABA and NMDA exposure up till DIV28. Nevertheless, it is important to characterise any iPSC-derived model and elucidate the

receptor profile at different days in culture prior to neurotoxicity testing as it has been shown that cellular properties can vary over time due to the maturation process of the cells (Odawara et al., 2014; Dage et al., 2014).

In conclusion, we show that human iPSC-derived neurons from different suppliers all form neuronal structures and develop spontaneous network activity. We also show that the cultures contain different neuronal subtypes, such as GABAergic and glutamatergic neurons.The human origin and short culture duration are major benefits of these commercial iPSCs, in particular for iCell neurons which are already spontaneously active following 4-6 DIV. However, the absence of bursting in these cells indicates that human iPSC-derived neuronal cultures may not yet be able to fully replace the rat primary cortical model. While addition of astrocytes and/or changes in culture conditions as well as additional characterisation, optimisation and toxicological validation are urgent pre-requisites, our data already indicate that human iPSC-derived neurons resemble primary cortical cultures when exposed to different physiological and pharmacological stimuli. Despite current limitations and disadvantages (high cost, absence of bursting, absence of astrocytes), human iPSC-derived neurons may enable animal-free neurotoxicity testing in the future and can already be used as a quick screening tool for chemical prioritisation and effect screening studies prior to animal studies or studies using primary cells.

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Conflict of interest statement
The authors declare that they have no conflict of interests.

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Tab. 1: Development of spontaneous neuronal activity in different human iPSC-derived neurons.

	DIV4-6				DIV7-9				
	MSR[1]	% active wells	% active electrodes		MSR[1]	% active wells	% active electrodes		n/N[2]
HIP	0.068	35.8	3.1		0.083	52.5	6.0		1917/120
DOPA.4U	0.214	40.0	5.9		0.240	48.4	7.6		1519/95
iCell	0.373	88.2	19.6		0.331	81.3	17.4		2301/144
Primary rat cortical cells	n.a.[3]	n.a.[3]	n.a.[3]		0.353	94.8	32.0		768/48

[1] Mean spike rate (MSR) in spikes/s.
[2] Total number of electrodes (n) derived from N wells used to calculate MSR, % active wells and % active electrodes. See methods for details.
[3]n.a., not applicable. Primary cortical cultures do not develop significant activity before DIV5-6.

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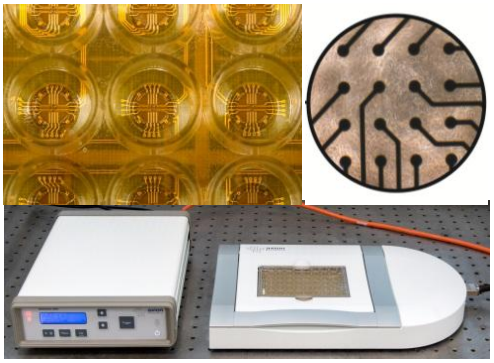

Fig. 1:Experimental set up for measurements of spontaneous electrical network activity
Human iPSC-derived neurons from different suppliers were cultured on the electrode grids in 48-well MEA plates (top left and right) for recording of neuronal activity using Axion's Maestro (bottom).

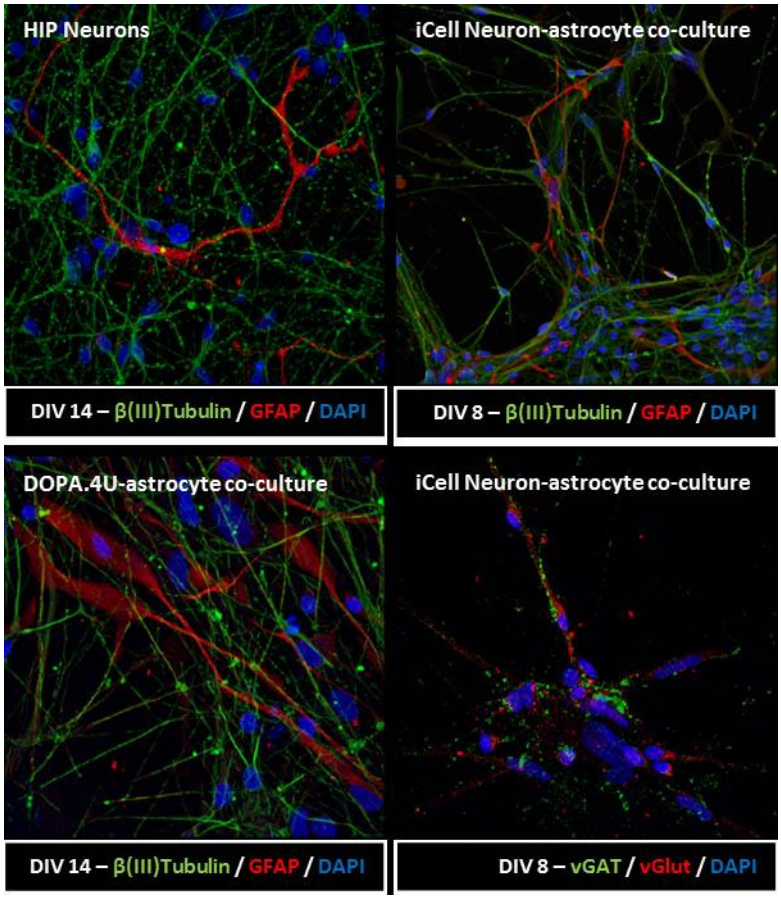

Fig. 2: Immunofluorescent staining of human iPSC-derived neurons
Different human iPSC-derived neurons were stained with β(III)tubulin (green) and GFAP (red) antibodies to identify the presence of respectively neurons and astrocytes (HIP® neurons, upper left; DOPA.4U® neurons, lower left; iCell neurons, upper right). Human iCell neurons were stained with vGAT (green) and vGluT (red) antibodies to demonstrate the presence of inhibitory (GABA-ergic) and excitatory (glutamatergic) neuronal contacts (lower right). Nuclei are stained with DAPI (blue).

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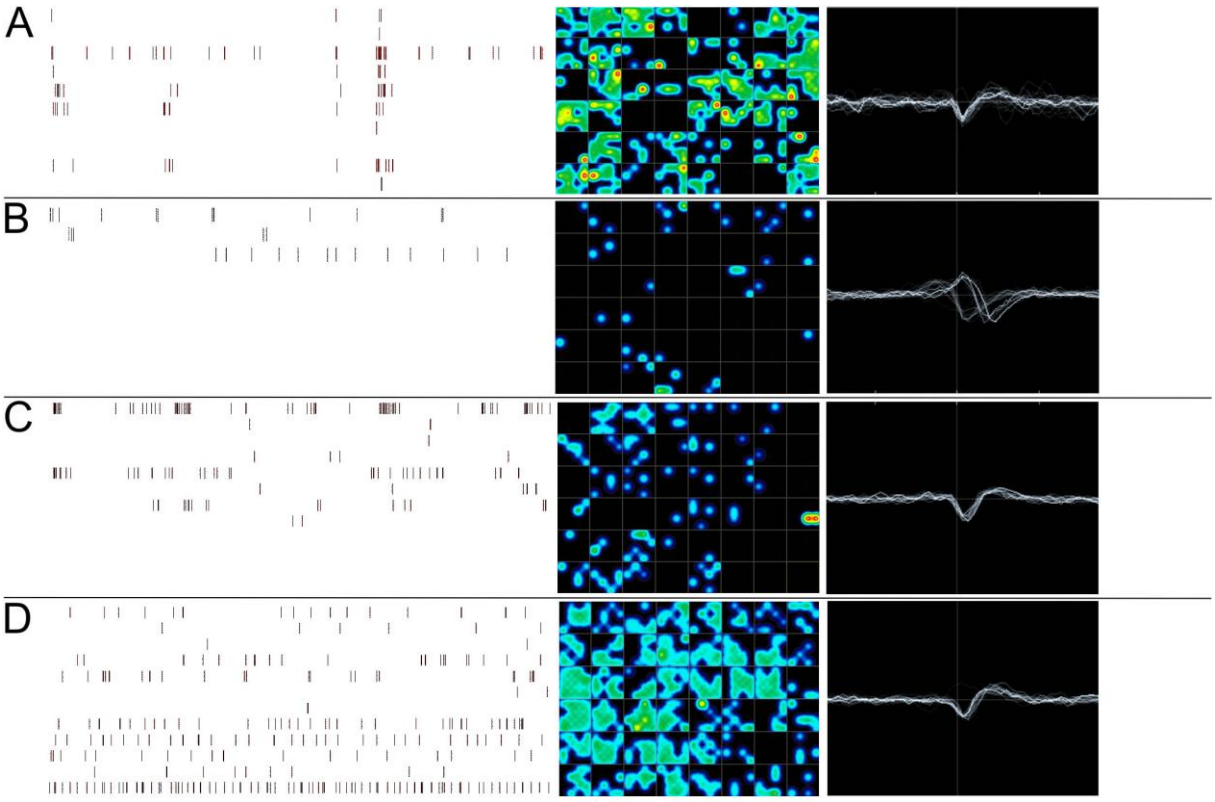

Fig. 3:Spike raster plot, activity heat maps and example traces of individual field potentials illustrating the degree and pattern of neuronal activity of the primary rat cortical culture
Spike raster plot (left; each row depicts one electrode in a representative example well, with every mark representing one spike in a 20s interval), activity heat maps (middle; degree of activity in a 48-well plate depicted in false colour) and example traces of individual field potentials (right; recorded at a single representative electrode) illustrating the degree and pattern of neuronal activity of the primary rat cortical culture (A), HIP neurons (B), DOPA.4U neurons (C) and iCell neurons (D) at DIV7-8.

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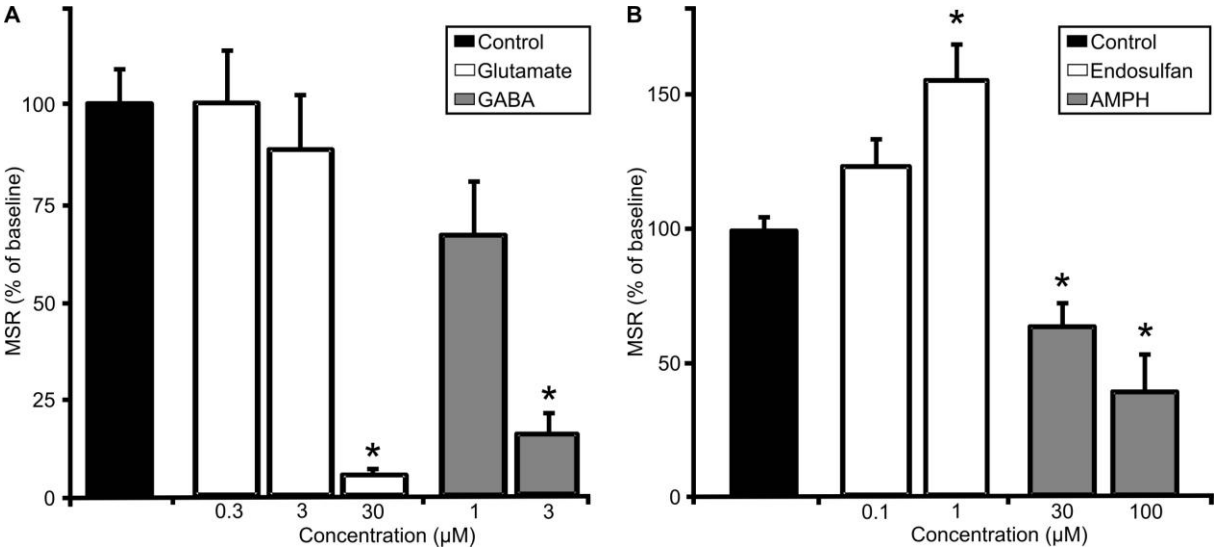

Fig. 4:Physiological (A) and pharmacological (B) modulation of spontaneous network activity of iCell neurons

At DIV7-8, human iCell neurons were challenged with glutamate (0.3-30 µM), GABA (1-3 µM), endosulfan (0.1 or 1 µM) or amphetamine (AMPH; 30 or 100 µM). Data are expressed in MSR as % of control; mean ± SEM from 3-20 wells (N).

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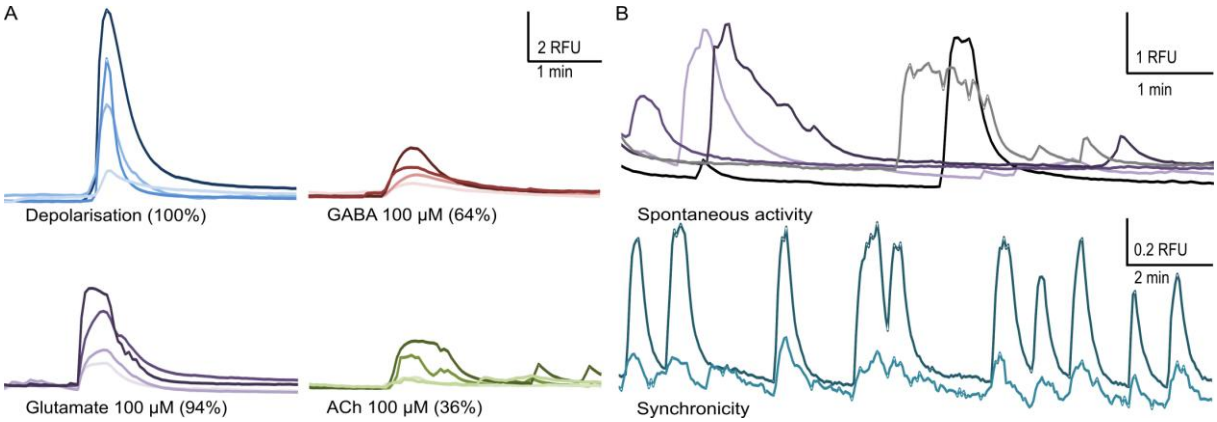

Fig. 5:Representative traces demonstrating the increase in $[Ca^{2+}]_i$observed in individual iCell neurons following exposure to different stimuli
(A). Percentages (in between brackets) indicate the fraction of cells responsive to the different stimuli (obtained from 21-57 individual cells in 3-4 independent experiments). Synchronicity and spontaneous activity were also observed (B).