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Do *in vitro* assays in rat primary neurons predict drug-induced seizure liability in humans?

Kreir M.[a,*], Van Deuren B.[a], Versweyveld S.[b], De Bondt A.[c], Van den Wyngaert I.[c], Van der Linde H.[a], Lu H.R.[a], Teuns G.[a], Gallacher D.J.[a]

[a] Preclinical Development and Safety, Discovery Sciences, Janssen Research and Development, Janssen Pharmaceutica NV, Beerse, Belgium
[b] Neuroscience Discovery, Janssen Research and Development, Division of Janssen Pharmaceutica NV, Beerse, Belgium
[c] Computational Sciences, Discovery Sciences, Janssen Research and Development, Janssen Pharmaceutica NV, Beerse, Belgium

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ABSTRACT

Drug-induced seizures contribute to the high attrition rate of pharmaceutical compounds in development. The assessment of drug-induced seizure liability generally occurs in later phases of development using low throughput and intensive *in vivo* assays. In the present study, we evaluated the potential of an *in vitro* assay for detecting drug-induced seizure risk compared to evaluation in rats *in vivo*. We investigated the effects of 8 reference drugs with a known seizurogenic risk using micro-electrode array (MEA) recordings from freshly-dissociated rat primary neurons cultured on 48-well dishes for 28 days, compared to their effects on the EEG in anesthetized rats. In addition, we evaluated functional responses and mRNA expression levels of different receptors *in vitro* to understand the potential mechanisms of drug-induced seizure risk. Combining the functional MEA *in vitro* data with concomitant gene expression allowed us to identify several potential molecular targets that might explain the drug-induced seizures occurring in both rats and humans. Our data 1) demonstrate the utility of a group of MEA parameters for detecting potential drug-induced seizure risk *in vitro*; 2) suggest that an *in vitro* MEA assay with rat primary neurons may have advantages over an *in vivo* rat model; and 3) identify potential mechanisms for the discordance between rat assays and human seizure risk for certain seizurogenic drugs.

1. Introduction

Within drug development, preclinical investigation includes focus on the potential of new medical entities (NME) that cause hazardous or adverse effects on different physiological systems within and above the expected therapeutic range of exposures. Drug-induced seizures (both convulsive and non-convulsive) are life-threatening adverse reactions, and have resulted in the withdrawal of several drugs from the market (Authier et al., 2016; Onakpoya et al., 2016) or termination of further drug candidate development throughout the various R&D stages. *In vivo* models have been developed and are widely used to study seizure liability. However, the general CNS safety study using modified Irwin test (Irwin, 1968) is generally conducted in the later phases of the drug development process (Kelly, 2004).

Seizure, or ictus, is defined as an uncontrolled electrical activity in the brain, which may produce a clinical convulsion, minor physical signs, thought disturbances, or a combination of these symptoms. Convulsions, tonic or clonic, are typically characterized by persistent or alternate contraction and spasm or relaxation of a set of voluntary muscles. Seizures with no external behavioral changes may also occur (Pitkänen et al., 2006). Such seizures can only be detected using typical EEG recordings, making detection of drug-induced abnormal neuronal activity in preclinical studies based solely on behavioral observations very difficult. Therefore, the facilitation of seizure risk identification of compounds early in the drug candidate selection process through assessment based on disturbances of neuronal activity *in vitro* that are indicative of seizure risk would represent an important and strategic step forward.

Defining seizures *in vitro* is very challenging and complex due to multiple pharmacological targets (Easter et al., 2009) and cellular mechanisms that need to be studied in a neuronal network. *In vitro* electrophysiology using hippocampal slice preparations from neonatal rodents has been a technique of choice to reproduce abnormal *seizure-like* activity and can detect the seizurogenic effects of a wide range of compounds. Single electrode and micro-electrode arrays (MEAs) for recording neuronal activity in hippocampal slices have also been

* Corresponding author.
E-mail address: mkreir@its.jnj.com (M. Kreir).

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successfully used in early drug development and safety pharmacology (Easter et al., 2007). However, the low throughput, the use of only a small portion of brain tissue, such as the hippocampus, and the use of large numbers of animals per study limit its utility and efficiency. Primary neuronal cultures from neurons dissociated from different regions of the central nervous system (CNS) have also long been used to study neurological mechanisms using the patch-clamp technique (Sakmann and Neher, 1984).

The combination of dissociated primary neurons with MEAs has more recently been introduced for neurotoxicity assessment (Gramowski et al., 2000). Recent studies have shown that re-aggregated cortex cell cultures allow exploration of network properties, while preserving the morphological, molecular and functional properties of the individual neurons (Chiappalone et al., 2006; Frega et al., 2012; Hondebrink et al., 2016). Moreover, another study demonstrated reproducibility and reliability of the MEA measurements across different laboratories using re-aggregated cells (Vassallo et al., 2016). However, some published studies investigated drug-induced neuronal toxicities by either analyzing the changes in the mean firing rate (Novellino et al., 2011; Vassallo et al., 2016), or by using a comprehensive set of parameters based on spike train characteristics (Hammer et al., 2015). Information resulting from MEA recordings can be utilized to classify compounds acting on different targets as reported previously by Gramowski et al., 2004 (Gramowski et al., 2004).

In the present study, to challenge these limitations of both *in vitro* and *in vivo* approaches used for safety pharmacology assessments of drug-induced seizure liability, we investigated the translatability between an *in vivo* assay in anesthetized rats and an *in vitro* assay using MEA recordings from rat primary neuronal cultures. To capture information on the electrical behavior of seizure-like patterns *in vitro*, we quantified different MEA parameters (or metrics) from the key combined bursting, network, and synchrony of the neuronal activity to establish potential characteristics for drug-induced seizure tendency *in vitro*. Because of the wide variety of molecular targets and off-targets of reference drugs with seizurogenic risk (Supplementary data Table S2), we investigated both functional responses of different receptors and their gene expression data to identify potential mechanisms and/or pathways that could be responsible for inducing seizures.

2. Materials and methods

2.1. Drugs

Chlorpromazine hydrochloride (CAS 69-09-0, purity ≥ 98%), amoxapine (CAS 14028-44-5, purity 99.5%, lot number BCBB6438V), pentylenetetrazol (CAS 54-95-5, purity ≥ 99%, lot number SLBF5034V), picrotoxin (CAS 124-87-8, purity ≥ 98%, lot number SLBN2682V), strychnine (CAS 57-24-9, purity ≥ 98%, lot number SLBP8489V), acetylcholine chloride (CAS 60-31-1, purity ≥ 99%, lot number BCBH3758V), epinephrine (CAS 52-43-4, purity ≥ 99%, lot number 044K1252), γ-Aminobutyric acid (GABA, CAS 56-12-2, purity ≥ 99%, lot number 38H4702), dopamine hydrochloride (CAS 62-31-7, purity ≥ 98%), pilocarpine hydrochloride (CAS 54-71-7, purity ≥ 98%, lot number MKBV5022V) and glutamate (CAS 6106-04-3, purity ≥98%, lot number BCBK6359V) were obtained from Sigma (Sigma-Aldrich, Diegem, Belgium). Serotonin hydrochloride (CAS 153-98-0, purity 98%, lot number 10158506) was obtained from Alfa Aesar (Alfa Aesar, Lancashire, UK). Histamine dihydrochloride (CAS 56-92-8, purity 99%, lot number A0249142) was obtained from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). Isoniazid (CAS 553-53-7, purity 97%, lot number FI246851501) was obtained from Carbosynth (Carbosynth Ltd., Berkshire, UK). Amitriptyline (CAS 50-48-6, purity > 95%) was obtained from Sequoia research products (Sequoia research products Ltd., UK).

2.2. Culture of rat primary neurons in vitro

Experiments were conducted using rat cortical cells that contain glutamatergic and GABAergic neurons and glia (Mundy and Freudenrich, 2000; Hogberg and Bal-Price, 2011). Primary neurons were freshly dissociated from embryonic E18-19 rat cortices as described previously in literature (Banker and Goslin, 1998) and 80,000 cells/well were plated onto 48-well MEA plates (Maestro system, Axion Biosystems). One day before plating the cells, each 48-well MEA plate was pre-coated with a polyethyleneimine (PEI) (0.1%) solution (Sigma), washed for four times with sterile distilled water and then allowed to dry overnight. On the day of plating, Laminin (20 μg/ml) (Sigma) was added to each plate which was then incubated for 1 h at 37 °C. Thereafter the neurons were cultured at 37 °C, 5% CO_2, 95% air atmosphere, in Neurobasal medium (Thermofisher cat. No. 21103-049) supplemented with 0.5 mM L-Glutamine (Thermofisher cat No 25030149) and 5% FBS (fetal bovine serum, from Thermofisher (cat No A3160802). The media was fully exchanged on the first day *in vitro* (DIV1) with Neurobasal media supplemented with glutamine and 2% B27 (Thermofisher cat. No 17504044) up to DIV5. From DIV5 onwards and every other day thereafter 50% of the media was changed with Neurobasal media containing 2% B27.

At DIV28, spontaneous neuronal activity obtained for 40 min in culture solution (Neurobasal + 2% B27) was defined as baseline. All the reference drug compounds were added at a single concentration per well (n = 8 per concentration within the same plate to avoid plate to plate variations) and plates were kept in the incubator (37 °C, 5% CO_2 and 95% O_2 atmosphere) for 60 min before being recorded for 40 min. Experiments with the neurotransmitters: GABA, epinephrine, serotonin, glutamate, dopamine and acetylcholine were analyzed after 3 min of exposure allowing us to capture transient effects of the neurotransmitters.

2.3. Anesthetized rats

All reported studies described here have been conducted in accordance with “The provision of the European Convention” on the protection of vertebrate animals which are used for experimental and other scientific purposes, and with “the Appendices A and B”, made at Strasbourg on March 18, 1986 (Belgian Act of October 18, 1991). Female Sprague-Dawley rats with a body weight range of 200 to 250 g were used in all experiments.

2.4. Anesthetic regime

Anesthesia was induced with a mixture of 5 mg/kg etomidate (Janssen Pharmaceutica NV, Beerse, Belgium) and 0.025 mg/kg fentanyl (Fentadon®, Eurovet Animal Health B.V., Bladel, the Netherlands) (van der Linde et al., 2011a). This mixture was injected *via* the tail vein. During the experiment, anesthesia was maintained with a continuous infusion of 10 mg/kg/h etomidate and 0.015 mg/kg/h fentanyl by i.v. infusion in the femoral vein. Just before the start of the infusion of drug compound, the rats received a subcutaneous injection of 0.5 mg/kg of the muscle relaxant succinylcholine (Janssen Pharmaceutica NV, Beerse, Belgium).

2.5. Experimental procedure

Rats were intubated with an endotracheal tube (Intramedic Polyethylene PE-200 tubing) for mechanical ventilation (Harvard Apparatus, Inspira asv) (tidal volume 3 ml, 60 resp/min). Three needle electrodes were placed on the cranium under the skin against the skull: two electrodes on the left and right and one reference electrode on the nose. Electrodes were connected to the Narcotrend (TM Monitor Technik, Germany) and a one lead EEG signal was monitored. Both left and right femoral arteries and veins were cannulated with Intramedic