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Role of Kv7.2/Kv7.3 and M_1 muscarinic receptors in the regulation of neuronal excitability in hiPSC-derived neurons

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ABSTRACT

The Kv7 family of voltage-dependent non-inactivating potassium channels is composed of five members, of which four are expressed in the CNS. Kv7.2, 7.3 and 7.5 are responsible for the M-current, which plays a critical role in the regulation of neuronal excitability. Stimulation of M_1 muscarinic acetylcholine receptor, M_1 receptor, increases neuronal excitability by suppressing the M-current generated by the Kv7 channel family. The M-current modulation via M_1 receptor is well-described in *in vitro* assays using cell lines and in native rodent tissue. However, this mechanism was not yet reported in human induced pluripotent stem cells (hiPSC) derived neurons. In the present study, we investigated the effects of both agonists and antagonists of Kv7.2/7.3 channel and M_1 receptor in hiPSC derived neurons and in primary rat cortical neuronal cells. The role of M_1 receptors in the modulation of neuronal excitability could be demonstrated in both rat primary and hiPSC neurons. The M_1 receptors agonist, xanomeline, increased neuronal excitability in both rat cortical and the hiPSC neuronal cells. Furthermore, M_1 receptor agonist-induced neuronal excitability *in vitro* was reduced by an agonist of Kv7.2/7.3 in both neuronal cells. These results show that hiPSC derived neurons recreate the modulation of the M-current by the muscarinic receptor in hiPSC neurons similarly to rat native neurons. Thus, hiPSC neurons could be a useful human-based cell assay for characterization of drugs that affect neuronal excitability and/or induce seizure activity by modulation of M_1 receptors or inhibition of Kv7 channels.

1. Introduction

The M-current is a non-inactivating voltage-dependent potassium current with slow kinetics in sympathetic neurons that have a dominant role in controlling membrane excitability (Marrion, 1997). The M-current plays an important role in a variety of neuronal and non-neuronal cells and its suppression increases responses to excitatory synaptic inputs (Moore et al., 1988; Xi-Moy and Dun, 1995). The channels that generate M-current in neurons are composed of heteromeric assembly of Kv7.2 or Kv7.3 with Kv7.3 subunits (Shapiro et al., 2000; Yus-Najera et al., 2003). Kv7.2, Kv7.3 and Kv7.3 are widely expressed and colocalize in the neocortex and the hippocampus, presenting a specific regionalized distribution (Cooper et al., 2000, 2001). Mutations in Kv7.2 and Kv7.3 subunits are associated with epileptic seizures in benign familial neonatal convulsions due to an attenuation of M-current which would prevent membrane repolarization and lead to hyperexcitability in the brain (Lerche et al., 2001; Steinlein, 2004). The M-current is strongly suppressed by the activation of muscarinic acetylcholine receptors (mACh receptors) (Brown and Adams, 1980). Five mACh receptor subtypes are identified, M_1-M_5 (Alexander et al., 2007). M_1, M_3 and M_5 receptors subtype couple predominantly through Gq/11 proteins to mobilize intracellular calcium and regulate multiple ion channel conductances including the voltage-dependent Kv7.2/3 channel (Hamilton et al., 1997) and calcium channel (Shapiro et al., 1999). Suppression of the active M-current by muscarinic agonists leads to membrane depolarization, rendering the cell more susceptible to firing (action potentials) thus potentially leading to seizures (Marrion, 1997). Therefore, seizures can be induced by drugs targeting the central cholinergic system such as pilocarpine (Turski et al., 1984) or carbachol (Snead, 1983) but also by directly targeting the potassium channels such as with linopirdine (Pena and Alavez-Perez, 2006) and diphenhydramine (Jang et al., 2010). The M_1 receptor is the predominant muscarinic acetylcholine receptor subtype in the cortex and the hippocampus (Oki et al., 2005) and is able to mediate the induction of seizures (Bymaster et al., 2003; Hamilton et al., 1997). The stimulation of M_1 receptor initiates seizures that progressively involve non-

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cholinergic systems. Although functional and phenotypic studies of M_1 muscarinic receptor and M-current to date have largely relied on *in vivo* assays with drug-induced epileptic models or knock-out models or *in vitro* assays using cell lines with heterologous expression of mACh receptors and Kv7.2/7.3, extrapolation of compound effects from those assays to clinical settings requires careful consideration.

Human induced pluripotent stem cells (hiPSCs) were recently established by transfecting human fibroblasts with the transcription factors Oct3/4, Sox2, Klf4, and c-Myc, which are expressed at high levels in embryonic stem cells (Takahashi and Yamanaka, 2006). Advancements in hiPSC technology have enabled differentiation of these cells into cultured neurons (Shi et al., 2012) which can be used as a tool to study pharmacological and toxicological testing, as well as the identification of new therapeutic targets (Izpisua Belmonte et al., 2009).

In the present study, we explored the functional expression of Kv7.2/7.3 channel and M_1 receptor in hiPSC derived neurons obtained from Ncardia (CNS.4U®). We evaluated the effect of several drugs targeting the voltage-gated potassium channels and the M_1 receptor using microelectrode arrays (MEAs) on neuronal cultures of hiPSC derived neurons and compared the compound effects observed with those recorded in the primary rat cortical neurons. Our results open opportunities to assess human-based cells with the hiPSC derived neurons for developing potential new drugs and for investigating mechanisms of drug-induced seizures via mACh receptors and Kv7 subunits.

2. Materials and methods

2.1. Drugs

Pilocarpine hydrochloride (CAS 54-71-7, purity ≥ 98%, lot number MKBV5022V), γ-Aminobutyric acid (GABA, CAS 56-12-2, purity ≥ 99%, lot number 38H4702), acetylcholine chloride (CAS 60-31-1, purity ≥ 99%, lot number BCBH3758V), picrotoxin (CAS 124-87-8, purity ≥ 98%, lot number SLBN2682V) and glutamate (CAS 6106-04-3, purity ≥ 98%, lot number BCBK6359V) were obtained from Sigma (Sigma-Aldrich, Diegem, Belgium). Flindokalner (BMS-204352) (CAS 187523-35-9, purity ≥ 98%), linopirdine dihydrochloride (DuP 996) (CAS 113165-57-3, purity ≥ 99%) and XE-991 dihydrochloride (CAS 122955-13-9, purity ≥ 99%) were obtained from Tocris Bioscience (Bio-Techne Ltd., UK). Xanomeline oxalate (CAS 141064-23-5, lot number 130201500x) and biperiden hydrochloride (CAS 1235-82-1, lot number 1109012031b) were obtained from Sequoia Research Products (Sequoia research products Ltd, UK).

2.2. Culture of rat primary neurons and human induced pluripotent stem cell-derived neurons in vitro

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). All experimental protocols were approved by the ethical committee of Janssen Pharmaceutica N.V. Experiments were conducted using cortical cells that contains glutamatergic and gabaergic neurons and glia (Hogberg and Bal-Price, 2011; Mundy and Freudenrich, 2000). Primary neurons were freshly dissociated from embryonic E18-19 rat cortices as described previously (Kreir et al., 2018; Valdivia et al., 2014) and plated onto 48-well MEA plates (Maestro system, Axion Biosystems). Human induced pluripotent stem cells, CNS.4U®, were obtained from Ncardia (Ncardia, Cologne, Germany) and cultured as per manufacturer’s instructions. The CNS.4U® is a heterogeneous group of neuronal and glial cells as per the manufacturer’s product specifications and contain ~30–40% glutamatergic, ~30–40% GABAergic and ~10% dopaminergic as well as ~10% astrocytes. One day before plating the cells, each 48-well MEA plate was pre-coated with a polyethyleneimine (PEI) (0.1%) solution (Sigma), washed 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 48-well plate which was then incubated for 1 h at 37 °C. Thereafter both types of 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 2% B27 (Thermofisher cat. No 17504044) for the rat cortical neurons and in the Neuro.4U® media supplemented with the neurosupplements provided by Ncardia for the hiPSC derived neurons. At DIV28, spontaneous neuronal activity obtained for 40 min in culture solution was defined as baseline. Compounds were added at a single dose per well (n = 8 per dose).

2.3. Data analysis

Data analysis was performed using AxIs software (Axion Biosystems Inc.) and GraphPad Prism (version 7.00; GraphPad Software Inc., San Diego, CA). Active electrodes, AEs, (16 electrodes per well) were defined as an electrode having an average of more than 6 spikes per min (0.1 Hz) (Wallace et al., 2015). An active well should have more than 15% active electrodes. All wells below this threshold were discarded upon these quality criteria. The threshold for the spike detection was ≥5.3x the standard deviation of the rms (root mean square) noise. Statistical analysis consisted of expressing the treatment ratio of exposed wells (percentage change between the baseline and the treatment) normalized to the treatment ratio of 100% in control experiments. Normalized treatment ratios of $n = 8$ wells were averaged per condition. Each well of the MEA served as its own control, and the changes in electrical activity elicited by the treatments were expressed as percent of that control activity and normalized to the wells treated with the vehicle control DMSO. The final concentration of DMSO added to each well was 0.1% (1 µl/ml), which did not alter the pH or the ionic concentration of the medium. Differences were determined using one-way ANOVA with Dunnett's correction; p values below 0.05 were considered significant. Data are expressed as means ± S.E.M.

2.4. Microarray assay and analysis

For microarray analysis, cells from neonatal rat cortical neurons at DIV28, dissociated cells from rat cortical and hippocampal brain tissues at the age of 6–7 weeks and hiPSC derived neurons CNS.4U® were lysed using RLT buffer (Qiagen) and RNA was extracted with the RNeasy 96 kit (p/n 74181 Qiagen). All microarray-related steps for target preparation, including the amplification of total RNA and labeling, were carried out as described in the GeneChip® WT PLUS Reagent kip (P/N 902281 Affymetrix 2017). Biotin-labeled target samples were hybridized to the GeneChip® Clariom_S_Rat_HT and the GeneChip® Clariom_S_Human_HT Array containing probes for almost 20k genes. Target hybridization was processed on the GeneTitan® Instrument according to the instructions provided in the User Guide for Expression Array Plates (P/N 702933). Images were analyzed using the GeneChip® Command Console Software (AGCC) (Affymetrix).

All microarray data were processed using the statistical computing R-program (R version 3.4.2; R Core Team, 2015) and Bioconductor tools (Gentleman et al., 2004). The gene expression values were normalized using RMA (Irizarry et al., 2003). Grouping of the individual probes into gene-specific probe sets was performed based on Entrez Gene using the metadata package (clariomsrathtrnentrezg and clariomshumanhthsentrezg, version 22.0.0) (Dai et al., 2005).

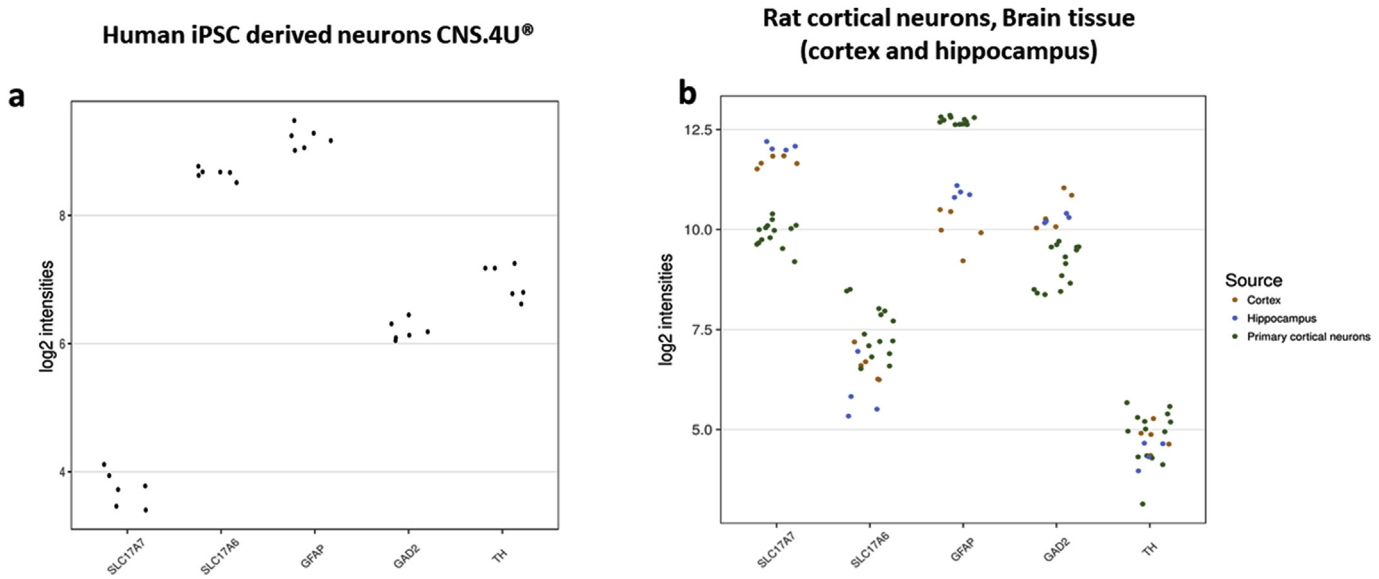

Fig. 1. Level of expression of synaptic and glial cell markers in a) human induced pluripotent stem cell derived neurons CNS.4U® and b) the primary rat cortical neuronal cell cultures and 2 brain tissues, i.e. rat cortex and rat hippocampus.

3. Results

3.1. Characterization of the human induced pluripotent stem cell derived neurons using microarray gene expression as compared to rat neuronal cells

We first characterized the level of expression of synaptic markers in human iPSC derived neurons and compared it to the expression in the rat cortical neurons. The hiPSC neurons showed expression of both glutamatergic and GABAergic vesicular genes vGLUT2 (Slc17a6) and GAD65 (Gad2) as well as expression of the astrocyte marker Gfap and the dopaminergic marker Th while vGLUT1 had a very low expression (Fig. 1a). The rat cortical neurons, as well as the rat cortex and hippocampus tissue, showed high expression levels of vGLUT1 (Slc17a7), Gfap and GAD65 while Th had a low expression level. vGLUT2 was also clearly expressed in all rat brain cells (Fig. 1b).

We then investigated which Kv7 subunits are expressed in rat cortical neurons and human iPSC derived neurons using microarray analysis. hiPSC neurons and rat cortical neurons were in culture for 28 days prior to their microarray analysis. In both rat cortical and hiPSC derived neurons Kv7.2 and Kv7.3 subunits were observed with relatively high expression levels. Kv7.4 and 7.5 showed a high expression level in rat cortical neurons while we observed low intensity in hiPSC derived neurons (Fig. 2). Kv7.1 showed low intensity expression in hiPSC derived neurons but little to no expression in rat primary cortical cells.

3.2. Development of spontaneous firing in hiPSC derived neuronal cultures on MEAs

To evaluate the electrical maturation of the hiPSC derived neurons, we recorded the spontaneous extracellular field activity over time (Fig. 3) using the MEAs. After 5 days in culture, the hiPSC exhibited spontaneous firing with an average of 0.17 ± 0.2 Hz per electrode, the number of bursts was 9.8 ± 0.1 bursts/min and the number of network bursts was 0.004 ± 0.02 bursts/min. The mean firing rate increased overtime to reach a maximum between DIV20 and DIV22, with 0.70 ± 0.3 Hz and 0.67 ± 0.3 Hz respectively. At DIV22 the number of bursts (Fig. 3B) was 25.4 ± 3.8 bursts/min and the network burst activity (Fig. 3C) was 1.06 ± 0.2 bursts/min. Between DIV28 and DIV30, the mean firing rate (0.38 ± 0.17 Hz and 0.38 ± 0.18 Hz) as well as the number of burst (15.6 ± 3 and 13.48 ± 6.6 burst/min) and the number of network bursts (1.94 ± 0.3 and 1.16 ± 0.8 bursts/min) showed a better synchronicity in bursts over the electrodes as shown by the raster plots in Fig. 3D. The average number of active electrodes and the number of electrodes participating in burst in each well over 8x 48-wells MEA (384 wells) was 6.98 ± 3.2 and 5.8 ± 2.8 respectively. In comparison, the rat cortical neurons at DIV28 exhibit in average a mean firing rate of 2.85 ± 1.8 Hz, a number of burst of 141 ± 75 bursts/min and a number of network bursts of 10.8 ± 5.1 bursts/min. All experiments for both the hiPSC derived neurons and the rat cortical neurons were performed at DIV28.

3.3. Electrophysiological responses of hiPSC derived neurons and rat cortical neurons to antagonists and agonists of potassium channels

We evaluated the function of the potassium channels in both rat and human cultured neuronal cells after 28 days in culture using microelectrode arrays and their functional response to a specific antagonist and agonist of the potassium ion channel mediating the M-current. Recordings of extracellular spontaneous action potentials organized in bursts and network bursts over the microelectrode arrays showed a dose-dependent increase in number of individual bursts in both rat cortical and hiPSC derived neurons in response to linopirdine, a blocker of Kv7.2/7.3 channels known to induce seizures (Fig. 4). Looking at the raster plots reported in Fig. 4A, there is a clear change in number of bursts of the hiPSC derived neurons after treatment with 1 μM linopirdine. The network bursts showed a significant increase in rat cortical neurons with 191% and 195% increase at 30 μM and 100 μM, respectively, whereas a maximum increase in activity of 220% was observed at 1 μM in hiPSC neurons. At 10 μM we observed a strong decrease in the number of network bursts in hiPSC derived neurons (Fig. 4B). The mean firing rate in rat cortical neurons did not significantly increase while the hiPSC derived neurons showed a significant increase (228.6%) at 1 μM linopirdine. At concentration > 30 μM we observed a strong inhibitory effect on the neuronal firing (MFR) of the hiPSC derived neurons (Fig. 4B).

Flindokalner, an agonist of Kv7 channels (Wulff and Zhorov, 2008) and Ca2 + -activated potassium-channels (BK channels) (Son et al., 2011) was used to see if opening of the potassium channels affected the spontaneous neuronal firing of the neuronal cells. Flindokalner did not significantly change MFR and the number of network bursts in hiPSC neurons, whereas we observed a significant decrease of neuronal activity at 3 μM in rat cortical neuronal cultures (Fig. 5).

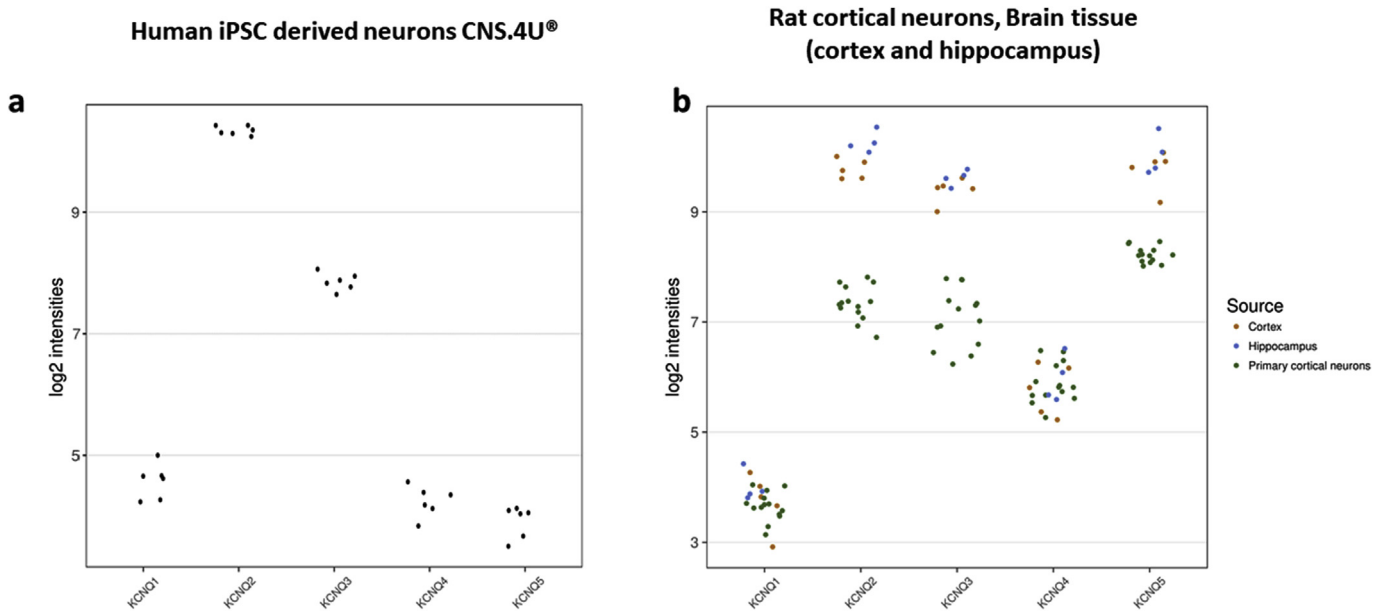

Fig. 2. Gene expression level of the potassium channel Kv7 (KCNQ) subunits present in a) human induced pluripotent stem cell derived neurons CNS.4U® and b) the primary rat cortical neuronal cell cultures compared to brain tissues from rat cortex and rat hippocampus.

3.4. Electrophysiological responses of hiPSC derived neurons and rat cortical neurons to antagonists and agonists of muscarinic acetylcholine receptor

Next, we evaluated the effect of muscarinic acetylcholine receptor agonists and antagonists, particularly those selective for the M_1 mACh receptor, which is involved in the regulation of the M-current. Biperiden, an antagonist of M_1 mACh receptor, and xanomeline, an agonist of M_1 mACh receptor, were used to modulate these receptors. The neuronal activity of rat cortical neurons in the presence of biperiden showed a decrease in mean firing rate and in the number of network bursts, whereas the mean firing rate did not change in hiPSC derived neurons, but the number of network bursts was strongly decreased (Fig. 6). At 0.1 µM, the number of network bursts showed a maximum decrease of 40% and 73% for the rat cortical and the hiPSC derived neurons, respectively.

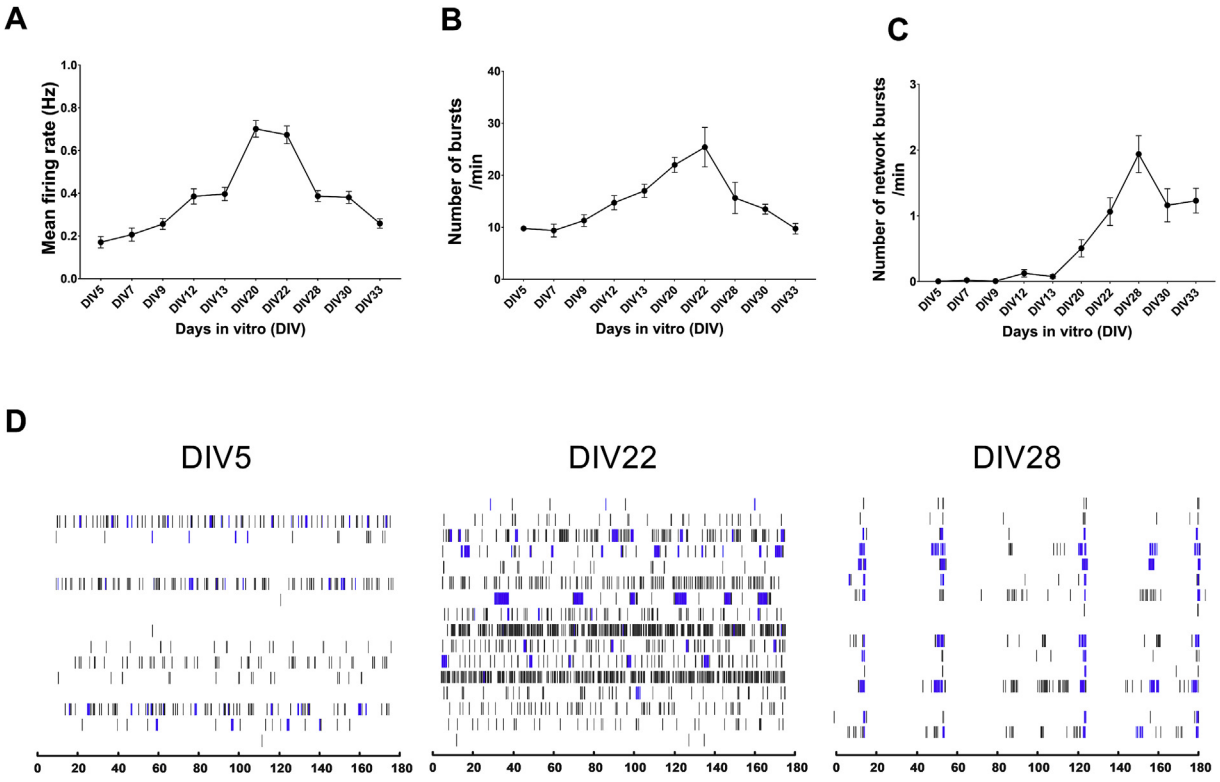

Fig. 3. Evolution of the spontaneous firing and bursting activity of the human iPSC derived neurons on the MEA over 33 days in culture. A. mean firing rate, B. number of bursts per min, and C. the number of network bursts per min. D. Spike raster plots for the 16 electrodes (each small vertical bar represents a spike, blue lines correspond to bursts, each line represents an electrode) over 180 s at DIV5, DIV22 and DIV28. Data are expressed as mean ± S.E.M. (n = 48).

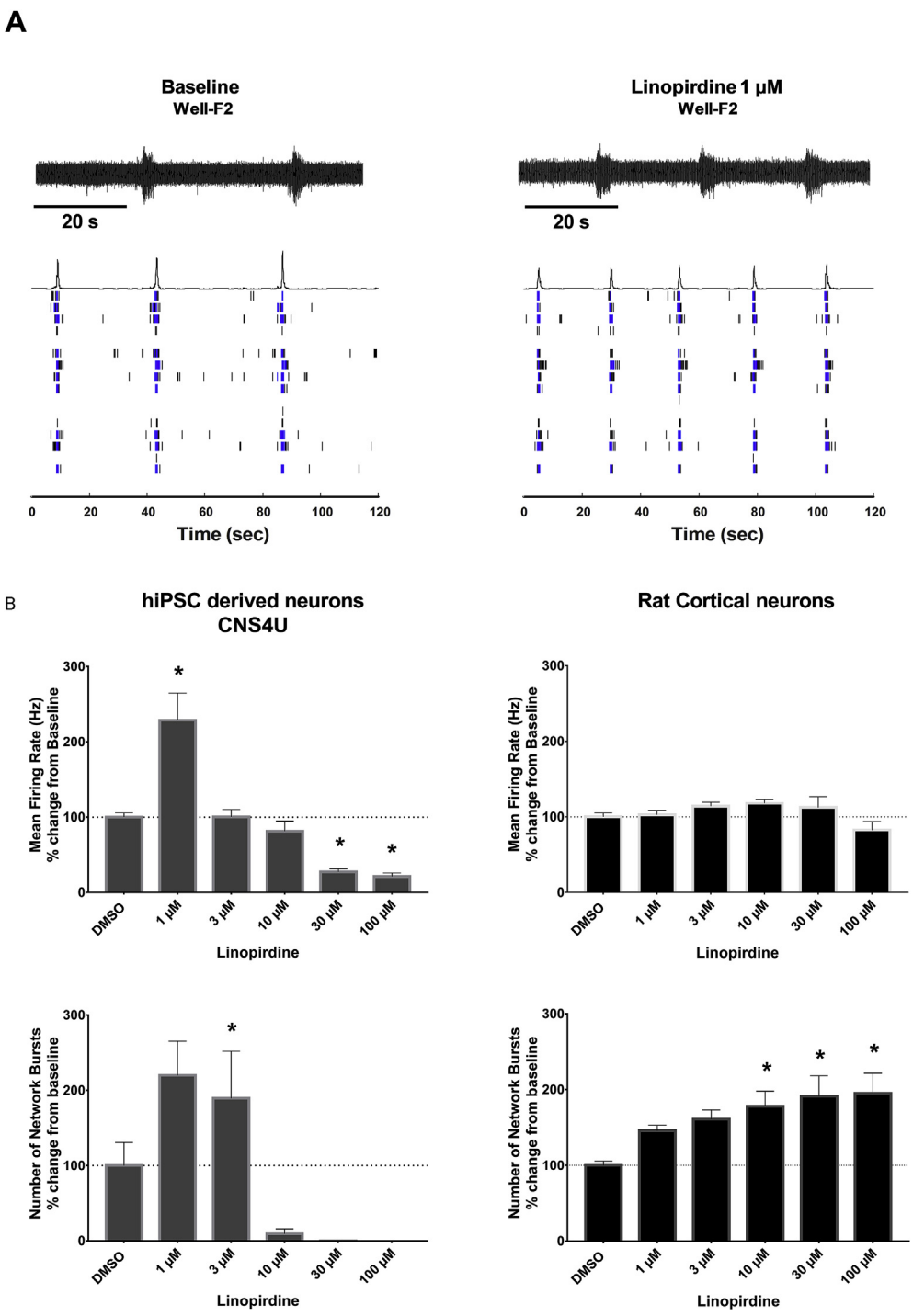

Fig. 4. Effects of linopirdine on neuronal activity neuronal activity of the hiPSC derived neurons and the rat cortical neurons. A. Linopirdine (1 µM) effect on the hiPSC derived neuronal network activity show an increase of activity in the raster plots (120 s of activity acquired from a representative hiPSC neuronal culture of 28 DIV). The number of network burst goes from 3 network bursts in the baseline to 5 in the same well after linopirdine treatment B. The mean firing rat (MFR) and the number of network bursts-of the hiPSC derived neurons (left) and the rat cortical neurons (right) measured with the MEA. Data are expressed as mean ± S.E.M. (n = 8). Statistical significance was determined using one-way ANOVA and Dunnett's test. *P < 0.05.

The agonist of M_1 the receptor, xanomeline (Fig. 7), showed an overall increase in the mean firing rate in both cell systems. The number of network bursts was strongly affected in hiPSC derived neurons with an increase of 227% at 0.3 µM compared to the rat primary cortical cells with an increase of 140% at 1 µM. The mean firing rate increased up to 1 µM (138%) in rat cortical neurons but firing activity decreased above 1 µM. In hiPSC derived neurons, we observed a significant increase of mean firing rate up to 0.3 µM (183%) and a decrease above 0.3 µM, close to or below the rate in DMSO treated neurons.

The responses to biperiden and xanomeline show that the M_1 receptor is functional in hiPSC derived neurons. Pilocarpine, a muscarinic acetylcholine receptor agonist activating both M_1 and M_3 receptors and known to induce seizures *in vivo,* was also tested. Pilocarpine showed an excitatory effect in both rats cortical and hiPSC derived neurons (Supplementary Fig. S1). Similarly, as with xanomeline, the effect of pilocarpine on hiPSC derived neurons occurred at lower concentrations compared to that on rat cortical neurons.

Overall, the hiPSC derived neurons responded to agonists and antagonists of M_1 mACh receptor and Kv7. However, they showed differences in sensitivity compared to the rat cortical neurons. Next, we investigated the impact of flindokalner, an agonist of the Kv7 channel, in another set of experiments on the xanomeline response in the neuronal cultures. Flindokalner inhibited the excitatory effect observed with xanomeline alone (Fig. 8), showing that opening the Kv7 channels can be used to reduce the individual firing and network burst rates.

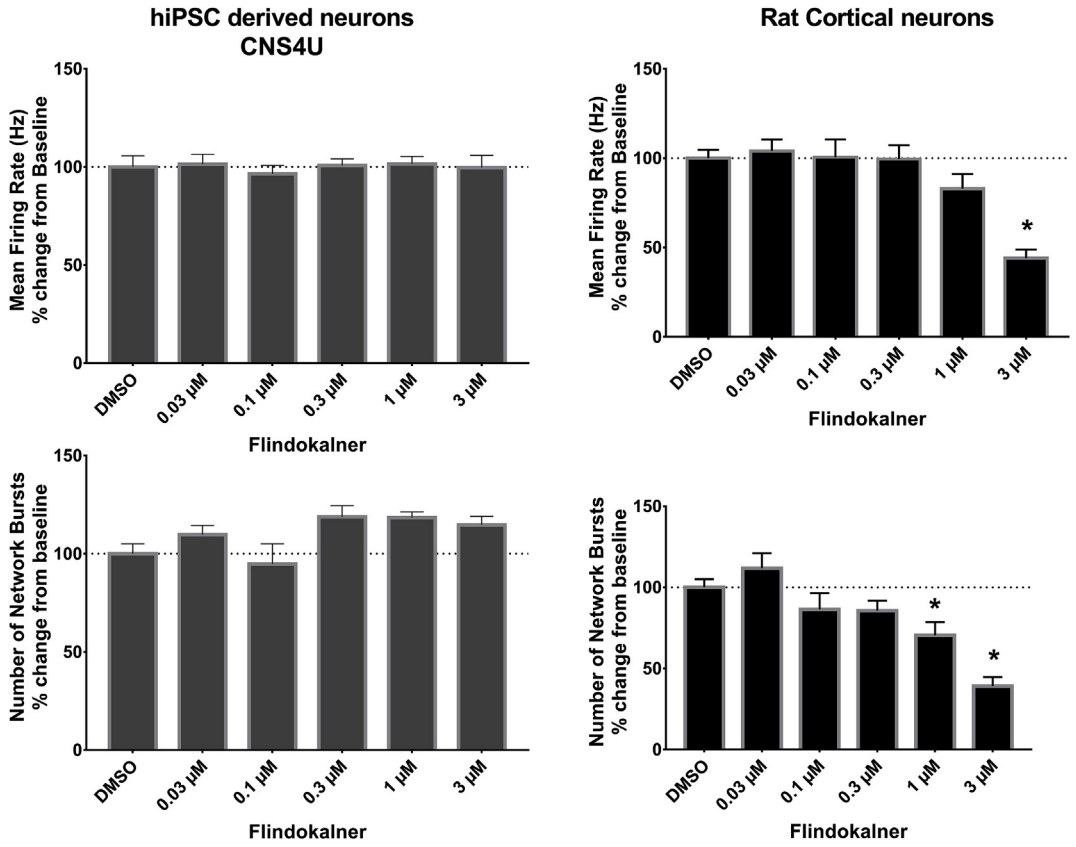

Fig. 5. Effects of flindokalner on neuronal activity -the mean firing rat (MFR) and the number of network bursts-of the hiPSC derived neurons (left) and the rat cortical neurons (right) measured with the MEA. Data are expressed as mean ± S.E.M. (n = 8). Statistical significance was determined using one-way ANOVA and Dunnett's test. *P < 0.05.

In addition, the importance of other key mechanisms linked to neuronal activities were studied using other compounds (XE-991, 4-aminopyridine, picrotoxin and phenytoin). The effects of neurotransmitters (glutamate, γ-aminobutyric acid and acetylcholine) were evaluated as well to further characterize the *in vitro* assay with human iPSC derived neurons (summarized in Table 1). The hiPSC derived neurons responded to glutamate and acetylcholine with an increase in neuronal activity through the maximal tested concentration. GABA evoked a concentration-dependent inhibition of the mean firing rate. The presence of GABA receptors was further evaluated using picrotoxin, a $GABA_A$ receptor antagonist, which changed the pattern of activity, characterized by an increase in bursts.

4. Discussion

The objective of this study was to explore the potential utility of human iPSC derived neurons as a model to examine the effects of compounds targeting KCNQ channels which play an important role in controlling membrane excitability (Marrion, 1997) and compounds targeting the muscarinic acetylcholine receptor, M_1, which can regulate the M-current generated through KCNQ channels (Hamilton et al., 1997). We also investigated potential differences in response between cultured primary cortical neurons (from Wistar rats) and hiPSC-derived neurons in terms of gene expression levels of specific neuronal markers and drug effects.

We initially characterized the expression levels of key gene markers indicative of neuronal function and potential responsiveness to physiological and pharmacological stimuli. We found that the hiPSC derived neurons provided by Ncardia (CNS4U®) express markers from glutamatergic, gabaergic and dopaminergic neurons as well as astrocytes markers. The gene expression levels of these markers showed differences between rat cortical neurons and hiPSC neurons. vGLUT1 is highly expressed in rat cortical neurons and rat brain tissues, whereas vGLUT2 is expressed at lower levels compared to vGLUT1. These two vGLUTs define the glutamatergic phenotype and display complementary distribution in the adult rat brain (Fremeau et al., 2004; Kaneko and Fujiyama, 2002). Their distribution is different, with vGLUT1 mainly expressed in the cerebral and cerebellar cortex and in the hippocampus, and vGLUT2 mainly expressed in the diencephalon, brainstem and spinal cord (Fremeau et al., 2002; Liguz-Lecznar and Skangiel-Kramska, 2007). However, Nakamura et *al.* reported that both vGLUTs are transiently co-expressed in the hippocampus and neocortex during development (Nakamura et al., 2005), which is observed in the rat cortical cell culture *in vitro*. The hiPSC derived neurons have low to no expression of vGLUT1 but high expression of vGLUT2. vGLUT2 was expressed together with the expression of the tyrosine hydroxylase (TH) marker, indicating the presence of dopaminergic neurons, and other markers such as HMX1, HMX2, NKx2-4, NKx6-1, which indicates that the hiPS cells used have a potential midbrain phenotype (supplementary data Fig. S2).

The electrical maturation of the hiPSC derived neurons was also characterized and compared to the rat primary neurons. The rodent exhibit earlier and stronger activity, specially the network activity which comes much later for the hiPSC derived neurons. At 14 days, the rat primary neurons exhibit already a mean firing rate of 2 Hz and a number of network busts of 7 bursts/min, which is higher than the hiPSC at DIV28. Similar observations were described by Napoli and Obeid (2016) between human primary and rodent primary neuronal cultures using MEA recordings.

Next, we evaluated the expression levels of the Kv7 subunits. The lack of expression of Kv7.1 was consistent between the two cell models and consistent with Kv7.1 expression throughout the body but absent

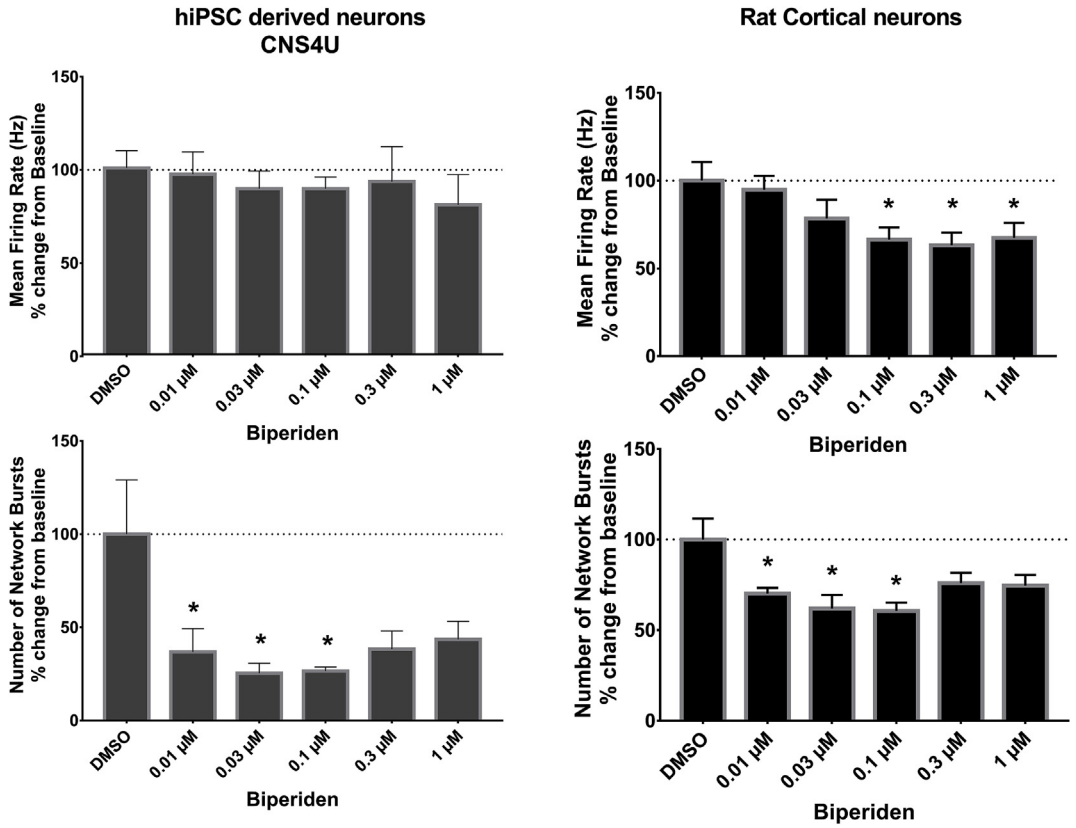

Fig. 6. Effects of Biperiden on neuronal activity -the mean firing rat (MFR) and the number of network bursts-of the hiPSC derived neurons (left) and the rat cortical neurons (right) measured with the MEA. Data are expressed as mean ± S.E.M. (n = 8). Statistical significance was determined using one-way ANOVA and Dunnett's test. *P < 0.05.

from the central nervous system (Cooper and Jan 2003). Kv7.2, 7.3 and 7.5 were highly expressed in the primary cells, in cortex tissues from rats and in hiPSC derived neuronal cells. KV7.2–7.5 subunits form homo- and heterotetrameric K^+ channels. KV7.2/3 contribute to the M-current and mutations in these channels cause benign familial neonatal convulsions (Jentsch, 2000). Kv7.4 was also expressed in rat neurons but not in hiPSC-derived neurons. The functionality of homo- or heterotetrameric channels with Kv7.4 subunits was not studied here but Shah et al. reported similar findings in rat hippocampal neurons where Kv7.4 was highly expressed but staining and single cell PCR showed the absence of Kv7.4 protein (Shah et al., 2002).

In electrophysiological recordings using the microelectrode arrays, linopirdine showed significant differences in dose-response in rat cortical and hiPSC-derived neurons. Linopirdine induces the release of a number of neurotransmitters, including acetylcholine (Aiken et al., 1996), by blocking KV7.2/7.3, leading to an excitatory effect in both neuronal cultures. This response is consistent with the role of Kv7 in the regulation of firing activity and spontaneous neuron population firing activity, as observed by the increase in network bursts. In rat cortical neurons linopirdine did not increase spontaneous firing (mean firing rate, MFR) but significantly increased the number of network bursts, thus defining the characteristic excitatory potential of linopirdine in the neuronal cultures. Inhibitory effects were observed at high concentrations of linopirdine (100 µM). On the other hand, linopirdine strongly increased both MFR and the number of network bursts in hiPSC-derived neurons at low concentrations but produced an inhibitory effect at 10 µM and higher. This concentration response documents a higher sensitivity of the hiPS cells to linopirdine. This increased sensitivity may be influenced by several factors such as a different expression levels of the KV7.2/7.3 channel and the calcium-activated potassium channel (BK), which can be inhibited by linopirdine at high concentrations (Schnee and Brown, 1998). BK channels participate to the regulation of neurotransmitter release and neuronal excitability, which can lead to the termination of neuronal firing when the channel is inhibited (Contet et al., 2016; Gu et al., 2007). When flindokalner, a potassium channel opener, was used at high concentrations the rat cortical neurons showed a decrease of activity, while no changes were observed in hiPSC-derived neurons. The effect observed in the rat cortical neurons might be the result of relative selectivity of flindokalner to activate KV7.3 homomeric channels, which are highly expressed in rat but have low expression in hiPS cells compared to KV7.3/7.5 and KV7.2/7.3 heteromeric channels (Dupuis et al., 2002; Korsgaard et al., 2005).

The modulation of the M-current generated by KV7.2/7.3 channels, and especially its suppression by M_1 receptor activation is well described in the literature (Brown and Adams, 1980; Gribkoff, 2003; Hamilton et al., 1997; Shapiro et al., 2000). Xanomeline, an agonist of M_1 receptor has a persistent effect on activation by interacting with more than one site on the receptor (Christopoulos and El-Fakahany, 1997). Xanomeline produced a significant increase of firing rate and network bursts in hiPSC-derived neurons and a slight increase in cultured rat cortical neurons. Inhibition of M_1 receptor led to an overall decrease in neuronal activity in both cell models. When xanomeline was used together with flindokalner, there was less increase in hiPSC neuronal activity compared to xanomeline alone. Flindokalner alone did not modify the overall neuronal activity but reduced the excitatory activity of xanomeline. Our results suggest that xanomeline indirectly inhibits the potassium current generated by KV7.2/7.3, which can be offset by flindokalner, as this latter opens KV7.2/7.3 channels. The hiPSC-derived neurons showed a higher response to xanomeline and to the combination of xanomeline and flindokalner compared to the primary rat cortical neurons. In the cortical neurons, the combination of compounds did not result in a stronger inhibition of the firing rate or network bursts compared to flindokalner alone. The signaling

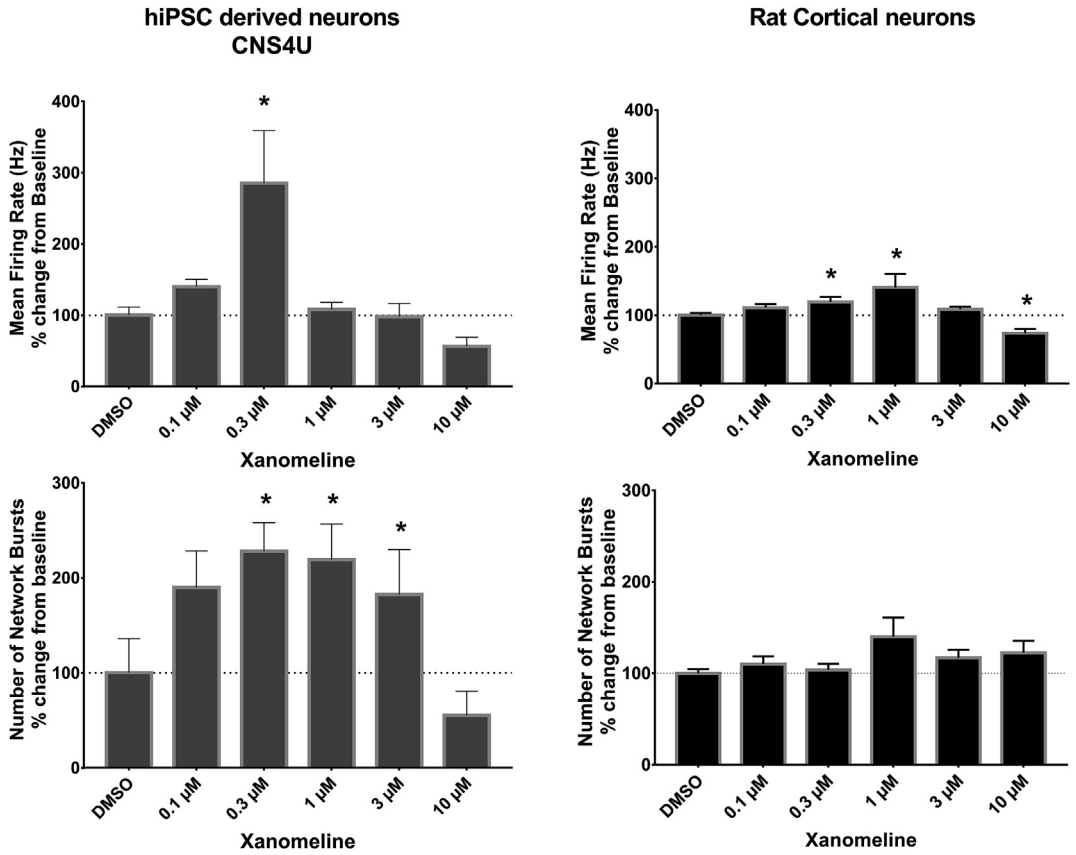

Fig. 7. Effects of xanomeline (M_1 agonist) on neuronal activity -the mean firing rat (MFR) and the number of network bursts of the hiPSC derived neurons (left) and the rat cortical neurons (right) measured with the MEA. Data are expressed as mean ± S.E.M. (n = 8). Statistical significance was determined using one-way ANOVA and Dunnett's test. *P < 0.05.

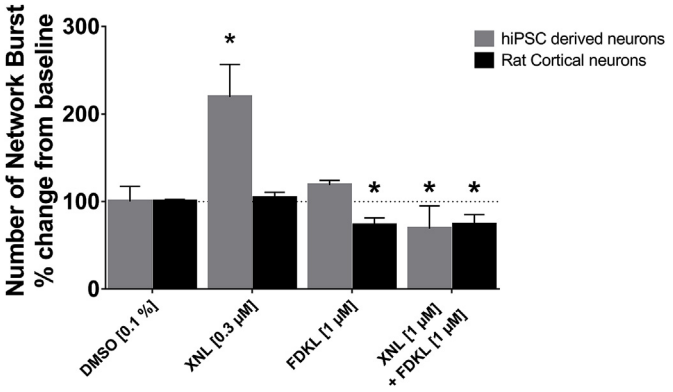

Fig. 8. Flindokalner suppresses the excitatory effect induced by xanomeline. Data are expressed as mean ± S.E.M. (n = 8). Statistical significance was determined using one-way ANOVA and Dunnett's test. *P < 0.05.

mechanism by which M_1 receptors suppresses the M-current which results in hyperexcitability involves the activation of Gq-coupled receptors. Xanomeline at concentrations above 1 µM has a prolonged engagement with the receptor and produces a persistent receptor activation (Christopoulos and El-Fakahany, 1997) leading to a sustained suppression of the M-current.

This suppression is due to the activation of phospholipase C (PLC), which consumes phosphatidylinositol 4,5-bisphosphate (PIP2) leading to its loss from the Kv7 subunits. Since PIP2 is required to prevent collapse of the ion-conducting pore, KV7.2/7.3 channel cannot transmit potassium ions without PIP2 (Suh and Hille, 2008). Another mechanism is mediated by protein kinase C (PKC), which phosphorylates the KV7.2 subunit to reduce PIP2 efficacy. This mechanism involves the A-kinase anchoring protein (AKAP79/150) which phosphorylates the KV7.2 subunit and dissociates calmodulin (CaM), thereby destabilizing KV7.2-PIP2 interaction, leading to the suppression of the M-current (Fig. 9) (Kosenko et al., 2012). Interestingly, retigabine, an agonist of Kv7 channels used for its antiepileptic properties, has been shown to be ineffective in opening the potassium channels suppressed by the muscarinic pathway (Kay et al., 2015). It is, therefore, possible that flindokalner effects involve other mechanisms for opening potassium channels or involves other ion channels compared to retigabine. Dupuis et al. showed that flindokalner can activate various Kv7 forming channels differently from retigabine suggesting another potential mechanism involved in opening the Kv7 channels (Dupuis et al., 2002; Schroder et al., 2001).

In conclusion, our data indicate that the human iPS cells used in these experiments are derived from neurons with many midbrain characteristics. While the hiPSC derived neurons form neuronal networks and show spontaneous activity and network bursting, they had overall a lower activity (5 fold lower) compared to the rat primary neurons, especially the network activity. Despite the limited number of compounds tested in our study, our data already showed different responses following exposure to drugs modulating M_1 receptor and Kv7 channels. Our data indicate that the hiPSC derived neurons had higher sensitivity for compounds targeting potassium channels and muscarinic acetylcholine receptors. Furthermore, astrocytes might play an essential role in the response to drugs and modulate the neuronal network activity and influence the sensitivity to drugs. Further pharmacology should be performed on hiPSC derived neurons containing or not astrocytes at different density (Tukker et al., 2018) to have models that could be a better tool for neurotoxicity.

Furthermore, antagonists of Kv7 channels and agonists of

Table 1
Overview of effects of activation and inhibitions of the key neuronal receptors and channels to induce neuronal excitability/inhibitory: the number of spikes and bursts, measured by microelectrode assay (MEA) on hiPSC derived neurons.

Compound	Target(s)	Effect on the number of spikes	Effect on the number of network bursts	n	Concentration (μM)	Free C_{max} (μM)	EC_{50}/IC_{50} (μM)
Linopirdine	Kv7.2/7.3	Increase	Increase	8	1–3	3.2	
Flindokalner	BKCa channels Kv7.2/7.3, Kv7.4/7.5	No effect	No effect	8	Up to 3	–	0.35
Biperiden	M_1 receptors	Decrease	Decrease	8	0.01–1	0.016	
Xanomeline	M_1/M_4 receptors	Increase	Increase	8	0.1–1	0.03–0.05	
Pilocarpine	M_1/M_3 receptors	Increase	Increase	8	0.01–0.3	0.03–0.55	
Glutamate	Glutamate receptors	Increase	Increase	8	0.3–10	–	1–2
GABA	GABA receptors	Decrease	Decrease	8	0.1–1	–	~1
Acetylcholine	ACh receptors	Increase	Increase	8	1–1000	–	1–10
Picrotoxin	GABA receptors	Increase	Increase	8	0.3–10	–	0.6–14
Phenytoin	Nav1.1/Nav1.3/Nav1.5	Decrease	Decrease	8	0.1–30	4–15	
XE-991	KV7.2/7.3	Increase	Increase	8	0.1–1	–	1.5
4-AP	Kv1.1 channel	Increase	Increase	8	0.1–10	0.025–0.075	

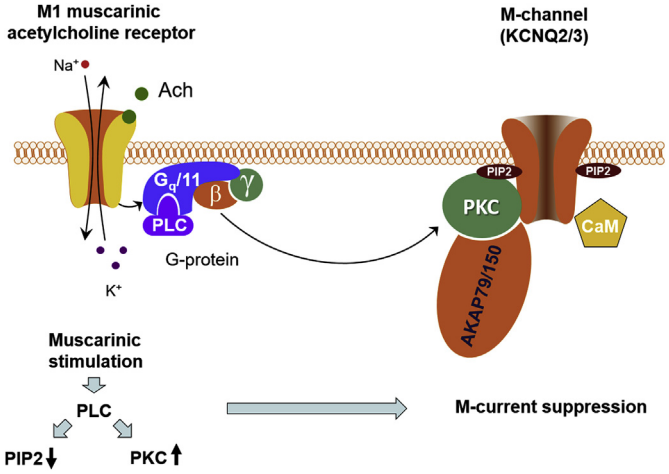

Fig. 9. Schematic representation of the signaling pathway discussed in the text that act on the Kv7.2/7.3 (KCNQ2/3) channels. Kv7.2/7.3 channels bind phosphatidylinositol-4,5-bisphosphate (PIP2), calmodulin (CaM) and A-kinase anchoring protein 79/150 (AKAP). PIP2 is required for Kv7 channel opening and AKAP facilitates the phosphorylation of Kv7 by protein kinase C (PKC). M_1 receptors couple to Gq/11 G-proteins and activate phospholipase-Cβ (PLCβ). This leads to hydrolysis of (PIP2) to produce inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG facilitate the activation of AKAP79/150 which recruit PKC leading to the depletion of PIP2 and thus the closing of KV7 channel currents.

muscarinic receptors have a high potential to induce seizures, which needs to be taken into consideration when developing drug candidates that may have off-target activity for these channels and receptors. Using both models we could re-create the excitatory effects of xanomeline, an agonist of M_1 receptor and linopirdine, an antagonist of Kv7 and BK channels, and attenuate these effects using a potassium channel opener, flindokalner. This suggests that the mechanism behind M-current suppression via muscarinic stimulation is conserved in the hiPSC-derived neurons. Further characterization involving a more extensive set of compounds could help us to cover the mechanism behind the M-current modulation via the M_1 receptor. Nevertheless, our data provide a proof of principle for the potential future use of hiPSC derived neurons for drug screening on specific target known to be expressed in such models, but this work could enable future relevant human based assay as a predictive model in replacement of *in vivo* assays. However, these human cells must express expected human receptors and respond pharmacologically to a wide range of compounds and conditions.

Author contributions

Conceived and designed the experiments: M.K., H.R.L., A.D.B., G.T. Performed the experiments: M.K., I.V.D.W. Analyzed the data: M.K., A.D.B. Wrote the manuscript: M.K., A.D.B., H.R.L., G.T., D.G. All authors reviewed the manuscript.

Competing financial interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2019.172474.

References

Aiken, S.P., Zaczek, R., Brown, B.S., 1996. Pharmacology of the neurotransmitter release enhancer linopirdine (DuP 996), and insights into its mechanism of action. Adv. Pharmacol. 35, 349–384.
Alexander, S.P., Mathie, A., Peters, J.A., 2007. Guide to receptors and channels (GRAC), 2nd edition (2007 revision). Br. J. Pharmacol. 150 (Suppl. 1), S1–S168.
Brown, D.A., Adams, P.R., 1980. Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. Nature 283, 673–676.
Bymaster, F.P., Carter, P.A., Yamada, M., Gomeza, J., Wess, J., Hamilton, S.E., Nathanson, N.M., McKinzie, D.L., Felder, C.C., 2003. Role of specific muscarinic receptor subtypes in cholinergic parasympathomimetic responses, in vivo phosphoinositide hydrolysis, and pilocarpine-induced seizure activity. Eur. J. Neurosci. 17, 1403–1410.
Christopoulos, A., El-Fakahany, E.E., 1997. Novel persistent activation of muscarinic M1 receptors by xanomeline. Eur. J. Pharmacol. 334, R3–R4.
Contet, C., Goulding, S.P., Kuljis, D.A., Barth, A.L., 2016. BK channels in the central nervous system. Int. Rev. Neurobiol. 128, 281–342.
Cooper, E.C., Aldape, K.D., Abosch, A., Barbaro, N.M., Berger, M.S., Peacock, W.S., Jan, Y.N., Jan, L.Y., 2000. Colocalization and coassembly of two human brain M-type potassium channel subunits that are mutated in epilepsy. Proc. Natl. Acad. Sci. U.S.A. 97, 4914–4919.
Cooper, E.C., Harrington, E., Jan, Y.N., Jan, L.Y., 2001. M channel KCNQ2 subunits are localized to key sites for control of neuronal network oscillations and synchronization in mouse brain. J. Neurosci. : Off. J. Soc. Neurosci. 21, 9529–9540.
Cooper, E.C., Jan, L.Y., 2003. M-channels: neurological diseases, neuromodulation, and drug development. Arch. Neurol. 60, 496–500.
Dai, M., Wang, P., Boyd, A.D., Kostov, G., Athey, B., Jones, E.G., Bunney, W.E., Myers, R.M., Speed, T.P., Akil, H., Watson, S.J., Meng, F., 2005. Evolving gene/transcript definitions significantly alter the interpretation of GeneChip data. Nucleic Acids Res. 33, e175.
Dupuis, D.S., Schroder, R.L., Jespersen, T., Christensen, J.K., Christophersen, P., Jensen, B.S., Olesen, S.P., 2002. Activation of KCNQ5 channels stably expressed in HEK293

cells by BMS-204352. Eur. J. Pharmacol. 437, 129–137.
Fremeau Jr., R.T., Burman, J., Qureshi, T., Tran, C.H., Proctor, J., Johnson, J., Zhang, H., Sulzer, D., Copenhagen, D.R., Storm-Mathisen, J., Reimer, R.J., Chaudhry, F.A., Edwards, R.H., 2002. The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. Proc. Natl. Acad. Sci. U.S.A. 99, 14488–14493.
Fremeau Jr., R.T., Voglmaier, S., Seal, R.P., Edwards, R.H., 2004. VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. Trends Neurosci. 27, 98–103.
Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5, R80.
Gribkoff, V.K., 2003. The therapeutic potential of neuronal KCNQ channel modulators. Expert Opin. Ther. Targets 7, 737–748.
Gu, N., Vervaeke, K., Storm, J.F., 2007. BK potassium channels facilitate high-frequency firing and cause early spike frequency adaptation in rat CA1 hippocampal pyramidal cells. J. Physiol. 580, 859–882.
Hamilton, S.E., Loose, M.D., Qi, M., Levey, A.I., Hille, B., McKnight, G.S., Idzerda, R.L., Nathanson, N.M., 1997. Disruption of the m1 receptor gene ablates muscarinic receptor-dependent M current regulation and seizure activity in mice. Proc. Natl. Acad. Sci. U.S.A. 94, 13311–13316.
Hogberg, H.T., Bal-Price, A.K., 2011. Domoic acid-induced neurotoxicity is mainly mediated by the AMPA/KA receptor: comparison between immature and mature primary cultures of neurons and glial cells from rat cerebellum. J. Toxicol. 2011, 543512.
Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., Speed, T.P., 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4, 249–264.
Izpisua Belmonte, J.C., Ellis, J., Hochedlinger, K., Yamanaka, S., 2009. Induced pluripotent stem cells and reprogramming: seeing the science through the hype. Nat. Rev. Genet. 10, 878–883.
Jang, D.H., Manini, A.F., Trueger, N.S., Duque, D., Nestor, N.B., Nelson, L.S., Hoffman, R.S., 2010. Status epilepticus and wide-complex tachycardia secondary to diphenhydramine overdose. Clin. Toxicol. 48, 945–948.
Jentsch, T.J., 2000. Neuronal KCNQ potassium channels: physiology and role in disease. Nat. Rev. Neurosci. 1, 21–30.
Kaneko, T., Fujiyama, F., 2002. Complementary distribution of vesicular glutamate transporters in the central nervous system. Neurosci. Res. 42, 243–250.
Kay, H.Y., Greene, D.L., Kang, S., Kosenko, A., Hoshi, N., 2015. M-current preservation contributes to anticonvulsant effects of valproic acid. J. Clin. Investig. 125, 3904–3914.
Korsgaard, M.P., Hartz, B.P., Brown, W.D., Ahring, P.K., Strobaek, D., Mirza, N.R., 2005. Anxiolytic effects of Maxipost (BMS-204352) and retigabine via activation of neuronal Kv7 channels. J. Pharmacol. Exp. Ther. 314, 282–292.
Kosenko, A., Kang, S., Smith, I.M., Greene, D.L., Langeberg, L.K., Scott, J.D., Hoshi, N., 2012. Coordinated signal integration at the M-type potassium channel upon muscarinic stimulation. EMBO J. 31, 3147–3156.
Kreir, M., Van Deuren, B., Versweyveld, S., De Bondt, A., Van den Wyngaert, I., Van der Linde, H., Lu, H.R., Teuns, G., Gallacher, D.J., 2018. Do in vitro assays in rat primary neurons predict drug-induced seizure liability in humans? Toxicol. Appl. Pharmacol. 346, 45–57.
Lerche, H., Jurkat-Rott, K., Lehmann-Horn, F., 2001. Ion channels and epilepsy. Am. J. Med. Genet. 106, 146–159.
Liguz-Lecznar, M., Skangiel-Kramska, J., 2007. Vesicular glutamate transporters VGLUT1 and VGLUT2 in the developing mouse barrel cortex. Int. J. Dev. Neurosci. 25, 107–114.
Marrion, N.V., 1997. Control of M-current. Annu. Rev. Physiol. 59, 483–504.
Moore, S.D., Madamba, S.G., Joels, M., Siggins, G.R., 1988. Somatostatin augments the M-current in hippocampal neurons. Science 239, 278–280.
Mundy, W.R., Freudenrich, T.M., 2000. Sensitivity of immature neurons in culture to metal-induced changes in reactive oxygen species and intracellular free calcium. Neurotoxicology 21, 1135–1144.
Nakamura, K., Hioki, H., Fujiyama, F., Kaneko, T., 2005. Postnatal changes of vesicular glutamate transporter (VGluT)1 and VGluT2 immunoreactivities and their colocalization in the mouse forebrain. J. Comp. Neurol. 492, 263–288.
Napoli, A., Obeid, I., 2016. Comparative analysis of human and rodent brain primary neuronal culture spontaneous activity using micro-electrode array technology. J. Cell. Biochem. 117, 559–565.
Oki, T., Takagi, Y., Inagaki, S., Taketo, M.M., Manabe, T., Matsui, M., Yamada, S., 2005. Quantitative analysis of binding parameters of [3H]N-methylscopolamine in central nervous system of muscarinic acetylcholine receptor knockout mice. Brain Res Mol Brain Res 133, 6–11.
Pena, F., Alavez-Perez, N., 2006. Epileptiform activity induced by pharmacologic reduction of M-current in the developing hippocampus in vitro. Epilepsia 47, 47–54.
Schnee, M.E., Brown, B.S., 1998. Selectivity of linopirdine (DuP 996), a neurotransmitter release enhancer, in blocking voltage-dependent and calcium-activated potassium currents in hippocampal neurons. J. Pharmacol. Exp. Ther. 286, 709–717.
Schroder, R.L., Jespersen, T., Christophersen, P., Strobaek, D., Jensen, B.S., Olesen, S.P., 2001. KCNQ4 channel activation by BMS-204352 and retigabine. Neuropharmacology 40, 888–898.
Shah, M., Mistry, M., Marsh, S.J., Brown, D.A., Delmas, P., 2002. Molecular correlates of the M-current in cultured rat hippocampal neurons. J. Physiol. 544, 29–37.
Shapiro, M.S., Loose, M.D., Hamilton, S.E., Nathanson, N.M., Gomeza, J., Wess, J., Hille, B., 1999. Assignment of muscarinic receptor subtypes mediating G-protein modulation of Ca(2+) channels by using knockout mice. Proc. Natl. Acad. Sci. U.S.A. 96, 10899–10904.
Shapiro, M.S., Roche, J.P., Kaftan, E.J., Cruzblanca, H., Mackie, K., Hille, B., 2000. Reconstitution of muscarinic modulation of the KCNQ2/KCNQ3 K(+) channels that underlie the neuronal M current. J. Neurosci. : Off. J. Soc. Neurosci. 20, 1710–1721.
Shi, Y., Kirwan, P., Livesey, F.J., 2012. Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. Nat. Protoc. 7, 1836–1846.
Snead 3rd, O.C., 1983. Seizures induced by carbachol, morphine, and leucine-enkephalin: a comparison. Ann. Neurol. 13, 445–451.
Son, Y.K., Choi, S.W., Jung, W.K., Jo, S.H., Jung, I.D., Park, Y.M., Choi, I.W., Sin, J.I., Shim, E.B., Kim, N., Han, J., Park, W.S., 2011. The inhibitory effect of Ca2+-activated K+ channel activator, BMS on L-type Ca2+ channels in rat ventricular myocytes. Life Sci. 89, 331–336.
Steinlein, O.K., 2004. Genes and mutations in human idiopathic epilepsy. Brain Dev. 26, 213–218.
Suh, B.C., Hille, B., 2008. PIP2 is a necessary cofactor for ion channel function: how and why? Annu. Rev. Biophys. 37, 175–195.
Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676.
Tukker, A.M., Wijnolts, F.M.J., de Groot, A., Westerink, R.H.S., 2018. Human iPSC-derived neuronal models for in vitro neurotoxicity assessment. Neurotoxicology 67, 215–225.
Turski, W.A., Cavalheiro, E.A., Bortolotto, Z.A., Mello, L.M., Schwarz, M., Turski, L., 1984. Seizures produced by pilocarpine in mice: a behavioral, electroencephalographic and morphological analysis. Brain Res. 321, 237–253.
Valdivia, P., Martin, M., LeFew, W.R., Ross, J., Houck, K.A., Shafer, T.J., 2014. Multi-well microelectrode array recordings detect neuroactivity of ToxCast compounds. Neurotoxicology 44, 204–217.
Wallace, K., Strickland, J.D., Valdivia, P., Mundy, W.R., Shafer, T.J., 2015. A multiplexed assay for determination of neurotoxicant effects on spontaneous network activity and viability from microelectrode arrays. Neurotoxicology 49, 79–85.
Wulff, H., Zhorov, B.S., 2008. K+ channel modulators for the treatment of neurological disorders and autoimmune diseases. Chem. Rev. 108, 1744–1773.
Xi-Moy, S.X., Dun, N.J., 1995. Potassium currents in adult rat intracardiac neurones. J. Physiol. 486 (Pt 1), 15–31.
Yus-Najera, E., Munoz, A., Salvador, N., Jensen, B.S., Rasmussen, H.B., Defelipe, J., Villarroel, A., 2003. Localization of KCNQ5 in the normal and epileptic human temporal neocortex and hippocampal formation. Neuroscience 120, 353–364.