Published in final edited form as:

J Pharmacol Toxicol Methods. 2016 ; 82: 109–114. doi:10.1016/j.vascn.2016.08.006.

Characterization of GABA_A receptor ligands with automated patch-clamp using human neurons derived from pluripotent stem cells

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Abstract

Introduction—Automated patch clamp is a recent but widely used technology to assess pre-clinical drug safety. With the availability of human neurons derived from pluripotent stem cells, this technology can be extended to determine CNS effects of drug candidates, especially those acting on the GABA_A receptor.

Methods—iCell Neurons (Cellular Dynamics International, A Fujifilm Company) were cultured for ten days and analyzed by patch clamp in the presence of agonist GABA or in combination with positive allosteric GABA_A receptor modulators. Both efficacy and affinity were determined. In addition, mRNA of GABA_A receptor subunits were quantified by qRT-PCR.

Results—We have shown that iCell Neurons are compatible with the IonFlux microfluidic system of the automated patch clamp instrument. Resistance ranging from 15–25 MΩ was achieved for each trap channel of patch clamped cells in a 96-well plate format. GABA induced a robust change of current with an EC₅₀ of 0.43 μM. Positive GABA_A receptor modulators diazepam, HZ166, and CW-04-020 exhibited EC₅₀ values of 0.42 μM, 1.56 μM, and 0.23 μM, respectively. The α2/α3/α5 selective compound HZ166-induced the highest potentiation (efficacy) of 810% of the current induced by 100 nM GABA. Quantification of GABA_A receptor mRNA in iCell Neurons revealed high levels of α5 and β3 subunits and low levels of α1, which is similar to the configuration in human neonatal brain.

Discussion—iCell Neurons represent a new cellular model to characterize GABAergic compounds using automated patch clamp. These cells have excellent representation of cellular GABA_A receptor distribution that enable determination of total small molecule efficacy and affinity as measured by cell membrane current change.

Keywords

CW-04-020; diazepam; GABA; GABA_A receptor; human-induced pluripotent stem cell; HZ166; iCell neuron; Ionflux; patch clamp

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Introduction

GABA (γ -aminobutyric acid) is the primary inhibitory neurotransmitter in the mammalian central nervous system. Most of the effects of GABA are generated through binding to the GABA_A receptor (GABA_AR). These receptors belong to a family of ligand-gated ion channels mediating fast synaptic transmission. At least twenty different GABA_AR subunits have been identified that assemble into pentameric structures in different combinations to form the native receptor (α_{1-6} , β_{1-3} , γ_{1-3} , δ , ρ_{1-3} , ϵ , θ , and π) (Barnard, et al., 1998). However, not all subunit combinations are present in cells (Jechlinger, Pelz, Tretter, Klausberger, & Sieghart, 1998; Nusser, Sieghart, & Somogyi, 1998). Most GABA_ARs *in vivo* are believed to be comprised of two α , two β , and one third subunit.

GABA_ARs are activated by binding directly to GABA and through allosteric modulation via benzodiazepines, barbiturates, steroids, anesthetics, convulsants, and many other drugs. Most of the receptor subtype-selective drugs identified so far interact with the benzodiazepine binding site of GABA_ARs. Because this site is located between α and γ subunits, drug selectivity is influenced strongly by their composition. There are six different α subunits and three different γ subunits, making possible 18 unique benzodiazepine binding sites. Most of the benzodiazepines mediate their effects in the brain predominantly by interacting with GABA_ARs composed of $\alpha 1\beta\gamma 2$, $\alpha 2\beta\gamma 2$, $\alpha 3\beta\gamma 2$, or $\alpha 5\beta\gamma 2$. These receptor subtypes mediate the diverse effects of benzodiazepine modulation. The $\alpha 4\beta\gamma 2$ and $\alpha 6\beta\gamma 2$ containing receptors, in contrast, do not appear to interact with many of the classical benzodiazepines such as diazepam, flunitrazepam, or clonazepam (Hevers & Luddens, 1998). The most abundant GABA_AR is $\alpha 1\beta 2\gamma 2$, with only a few brain regions lacking this receptor (Sieghart & Sperk, 2002). Benzodiazepines have been hailed as prototypic antianxiety agents, and although there are many alternatives to treating anxiety, none have matched the high efficacy and the rapid onset of these drugs (Basile, Lippa, & Skolnick, 2004). Furthermore, more than 50 years of clinical experience has demonstrated their extremely low degree of toxicity. However, secondary effects of these drugs have caused concerns with long-term treatment of anxiety disorders. These effects include sedation, anterograde amnesia, development of tolerance, and withdrawal symptoms (Ballenger, 2001). Extensive studies using gene-knockout techniques have determined that α subunits play a vital role in the diverse physiological effects of benzodiazepines. It has been revealed that $\alpha 1\beta 3\gamma 2$ containing receptors mediate sedative, amnesic and anticonvulsant actions of benzodiazepines and have high affinity for the classic scaffolds like diazepam (McKernan, et al., 2000). These receptors account for 60% of all GABA_ARs in the brain. In contrast, the $\alpha 2\beta 3\gamma 2$ GABA_AR is a highly specific target for the development of selective anxiolytic drugs. Representing only 15-20% of the total population of diazepam-sensitive GABA_ARs, selective modulators should be devoid of the major side-effects that plague the classic benzodiazepine anxiolytics (Rudolph, et al., 1999; Rudolph, Crestani, & Mohler, 2001). During the development of new subtype-selective compounds, it has been observed that high affinity to a particular GABA_AR is less important than functional efficacy. Thus, subtype-selective potentiation of GABA-induced current by a positive GABA_AR modulator is indicative of GABA_AR selectivity (Wafford, Whiting, & Kemp, 1993). In turn, subtype-

selective GABA_AR ligands might enable identification of the role of discrete combinations of GABA_ARs in the brain.

As an alternative to the study of CNS-derived neuronal cells, we have applied human-induced pluripotent stem cell (hiPSC) neurons as a model. These human neurons are a consistent cell population expressing primarily GABAergic and glutamatergic receptors, but have also been used to measure voltage-dependent Na⁺ and K⁺ channels (Becker, et al., 2013). The relative expression of GABA_AR subunits in iCell Neurons was determined by qRT-PCR. Following earlier work (Haythornthwaite, et al., 2012) that described patch clamp responses to GABA and antagonist bicuculline, we now present GABA-induced current responses in the presence and absence of subtype-selective GABA_AR modulators to determine their overall electrophysiological responses in iCell Neurons.

Methods

Cells

iCell Neurons (Cellular Dynamics International, a Fujifilm Company) were provided as cryopreserved single-cell suspensions in 1.5 mL cryo-vials containing 2.5 million plateable cells. Cells were thawed and plated in a 6-well plate per manufacturer's instructions. Poly-L-ornithine (Sigma-Aldrich) and laminin (Sigma-Aldrich) were used to provide the base layer for cell attachment to the plate (Costar). Cells were incubated at 37°C and 5% CO₂ and maintained for 10 days with exchange of 50% of the medium every 3 days. Cells were washed with Dulbecco's phosphate-buffered saline without Ca²⁺ and Mg²⁺ (Life Technologies) and cell dissociation was performed using 1X TrypLE Select (Invitrogen). Prior to an automated patch clamp experiment, cells were centrifuged at 380 × g for 5 minutes and resuspended gently in extracellular solution. Centrifugation and resuspension with fresh extracellular solution was repeated two more times before dispensing cells into the plate (8×10⁵ cells/ml).

Patch Clamp Solutions

The intracellular solution (ICS) contained (mM): 50 KCl 10 NaCl, 60 KF, 20 EGTA, 10 HEPES, pH 7.2 with KOH. The extracellular solution (ECS) contained (mM): 140 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 5 D-glucose monohydrate, and 10 HEPES, pH 7.4 with NaOH.

Compounds

A 10 mM stock solution of GABA (Sigma-Aldrich) in water was diluted in ECS to appropriate concentrations for use. HZ166 (Di Lio, et al., 2011) and CW-04-020 (PZ-II-029) (Varagic, et al., 2013) were provided by James Cook as 10 mM DMSO stocks. Diazepam (Sigma-Aldrich) was dissolved as a 10 mM solution in DMSO and diluted to appropriate concentrations in ECS for use.

Automated patch-clamp electrophysiology

Measurements were performed with the IonFlux instrument (Molecular Devices, Sunnyvale, CA), which utilizes microfluidic compound delivery on timescales below 100 ms, facilitating fast activating ligand gated ion channel measurements. Using this platform, a

large number of cells (20 per trap channel) can be held under voltage clamp and exposed to a series of compound concentrations within a short time period in parallel across a plate. Continuous recording coupled with fast solution exchange enables high-throughput. The IonFlux 16 plate layout consists of units of twelve wells; two wells contain intracellular solution (cytosolic), one contains cells diluted in extracellular solution, eight contain the compounds of interest diluted in extracellular solution, and one well is for waste collection. Cells are captured from suspension by applying suction to microscopic channels in ensemble recording arrays. Once the array is fully occupied, the applied suction breaks the cell membranes of captured cells, establishing a whole cell voltage clamp. For compound applications, pressure is applied to the appropriate compound wells, introducing the compound into the extracellular solution that is rapidly flowing over the cells. To record GABA_AR-mediated currents, cell arrays were voltage clamped at a holding potential of -80 mV. IonFlux software (IonFlux App) was used for data acquisition and exported into Excel (Microsoft) for data analysis. The data were uploaded to GraphPad Prism 5 (GraphPad Software) for visualization, determination of standard deviations, and non-linear regression analysis using $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$ for concentration-dependent response curves.

qRT-PCR

iCells were homogenized using the QIAshredder (Qiagen) and RNA isolated with the RNeasy kit (Qiagen). Total RNA was quantified with a Tecan Infinite M1000 plate reader (Tecan) in a UV-Star 384-well plate (Greiner Bio-One). A QuantiFast SYBR Green RT-PCR Kit (Qiagen) was used for the real time PCR following manufacturer's recommendations. Primers used in these studies were as follows: GAPDH FP 5'-ACCACAGTCCATGCCATCAC-3', GAPDH RP 5'-TCCACCACCCTGTTGCTGTA-3'; GABRA1 FP 5'-GGATTGGGAGAGCGTGTAACC-3'; GABRA1 RP 5'-TGAAACGGGTCCGAAACTG-3'; GABRA2 FP 5'-GTTCAAGCTGAATGCCCAAT-3', GABRA2 RP 5'-ACCTAGAGCCATCAGGAGCA-3'; GABRA3 FP 5'-CAACTTGTTTCAGTTCATTCATCCTT-3', GABRA3 RP 5'-CTTGTTTGTGTGATTATCATCTTCTTAGG-3'; GABRA4 FP 5'-TTGGGGGTCCTGTTACAGAAG-3', GABRA4 RP 5'-TCTGCCTGAAGAACACATCCA-3'; GABRA5 FP 5'-CTTCTCGGCGCTGATAGAGT-3', GABRA5 RP 5'-CGCTTTTCTTGATCTTGGC-3'; GABRA6 FP 5'-ACCCACAGTGACAATATCAAAAGC-3', GABRA6 RP 5'-GGAGTCAGGATGCAAAACAATCT-3'; GABRB3 FP 5'-CCGTTCAAAGAGCGAAAGCAACCG-3', GABRB3 RP 5'-TCGCCAATGCCGCCTGAGAC-3'; GABRG2 FP 5'-AACATGGTGGGGAAAATCTG-3', GABRG2 RP 5'-GGCAGGAGTGTTTCATCCATT-3'; Real-time rt-PCR was carried out on a Mastercycler (Eppendorf). The Ct method was used to normalize Cts of target genes to housekeeping gene GAPDH. Standard deviations were calculated from two independent experiments performed in triplicates.

Results

For the automated patch-clamp experiment, 320 iCell Neurons were trapped successfully in the sixteen trap channels of a 96 well IonFlux 16 plate, which enabled simultaneous recording of eight individual recordings in duplicate (Spencer, et al., 2012). Each recording is the average of 20 individually patched clamped cells. The cell occupancy of the individual microchannels of each trap channel was verified by microscopy for all plates after the recording. The cell seal resistance ranged between 15-25 M Ω for four IonFlux plates as depicted in Figure 1.

During the beginning of the experiment, patched clamped iCell Neurons were treated with extracellular solution (ECS) using eight individual microfluidic systems (ensembles) arranged in a 96 well IonFlux 16 plate. The cells were voltage-clamped at a holding potential of -80 mV and a stable current base line was established during 60 seconds (Supplementary Material, Figure S1). Subsequently, patched clamped iCell Neurons were treated with increasing concentrations of GABA dissolved in ECS from wells that are connected by microfluidic channels to two trap channels per ensemble (Spencer, et al., 2012). Each GABA application lasted for 3000 ms. During that time, negative current increased, reached saturation followed by desensitization (Figure 2A). A rapid negative current decrease was observed once GABA was washed away with ECS by closing the GABA-containing microfluidic channel. A change of negative current relative to the baseline was observed at 0.14 μ M GABA. The maximum negative current was achieved with GABA at 1 μ M and greater. The success rate of the assays, defined as detectable negative current of more than -500 pA for the highest concentration of GABA, was 100%. Seven increasing concentrations of GABA were used to determine the electrophysiological EC₅₀ value of GABA for iCell Neurons (Figure 2B). The experiment was carried out with sixteen independent ensemble trap channels. The EC₅₀ value of 0.43 ± 0.19 μ M was determined using non-linear regression. Furthermore, a single cell electrophysiology experiment was carried out using a GigaOhm Seal Plate in combination with the IonFlux. The success rate for this plate dropped to 25%, however, the resistance for iCell Neurons increased to 100 M Ω . The individual current traces for different GABA concentrations are depicted in Figure 2C. Similar to the multi-cell experiments, negative current increased and saturated during three second application periods of GABA followed by rapid decrease of negative current during the washout period. The overall current changes were smaller than those recorded for multi-cell experiments (Figure 2A).

The reproducibility of current change for 100 nM GABA (EC₃) was established to determine the electrophysiological effects of positive allosteric GABA_AR modulators (Figure 2D). It has been reported that GABA binds at the interface of α and β subunits in GABA_ARs (Sigel, Baur, Kellenberger, & Malherbe, 1992), whereas the class of positive allosteric GABA_AR modulators investigated here bind between the α and γ subunits (Sigel & Buhr, 1997).

GABA has to be present at sub EC₅₀ concentrations to observe electrophysiological responses of GABA_ARs in the presence of GABA_AR modulators. GABA concentrations reported for determining current changes of positive allosteric GABA_AR modulators range

between EC₃ and EC₂₀ concentrations. It was demonstrated here that repeated three second applications 100 nM GABA (EC₃) resulted in a consistent negative current change when applied six consecutive times (Figure 2D).

In addition, repeated application of 100 nM GABA for three seconds preceded the application of increasing concentrations of positive allosteric GABA_AR modulators in the presence of 100 nM of GABA as part of the automated patch clamp protocol to characterize the electrophysiological affinity and efficacy of GABA_AR modulators for iCell Neurons (Figure 2). The average negative current change in the presence of 100 nM GABA equals 100% of control current at GABA EC₃ for Figures 3B, 3D, and 3F. For compound application, GABA_AR modulators were dissolved in ECS with a maximum of 0.3% DMSO. At a concentration of 0.41 μM diazepam in the presence of GABA a significant negative current change was observed (Figure 2A). Diazepam between 1.23 μM and 11.1 μM resulted in a higher but similar negative current change that enabled affinity and efficacy determination. The EC₅₀ value calculated for diazepam for iCell Neurons was 0.42 ± 0.12 μM with a potentiation of 593% relative to the current observed for 100 nM GABA. The affinity of diazepam among GABA_A receptors bearing different α-subunits using HEK293 cells transfected with GABA_AR subunits decreased from α1>α5>α2>α3, whereas the efficacy for the same assay, though very similar, decreased from α2>α1>α5>α3 (Rabe, Kronbach, Rundfeldt, & Luddens, 2007). Interestingly, 33 μM diazepam induced a second component of potentiation, as reported earlier for α1β2γ2 transfected oocytes, due to the presence of two components of potentiation in GABA_A receptors through binding of α1β2γ2 and α1β2 (Walters, Hadley, Morris, & Amin, 2000). HZ-166 is a selective anxiolytic compound with less efficacy to α1 subtype bearing GABA_ARs. The selectivity pattern of this compound is α5>α2>α1>α3 for affinity and α3>α2>α5>α1 for efficacy (Fischer, et al., 2010). Investigations with iCell Neurons using the IonFlux system showed a slow but steady increase of negative current starting at 0.14 μM HZ-166 (Figure 3C). Saturation of negative current signals was less pronounced at higher concentrations of HZ-166, which might be caused by occupation of different populations of GABA_ARs as observed for diazepam. The calculated EC₅₀ for the most sensitive GABA_A receptor was 1.56 ± 0.83 μM at a potentiation of 810% relative to the current change observed for 100 nM GABA (Figure 3D). Finally a recently discovered α6-selective GABA_AR modulator CW-04-020 (PZ-II-029) was investigated (Varagic, et al., 2013), demonstrating a significant increase in negative current at 0.14 μM (Figure 3E). Negative current changes did not change significantly at concentrations higher than 1.23 μM and resulted in an EC₅₀ of 0.23 ± 0.07 μM (Figure F). The potentiation based on the negative current observed at 100 nM GABA was 480%.

The expression of GABA_AR subunits in iCell Neurons was determined by qRT-PCR (Figure 4). A moderate amount of γ2 mRNA was identified, which combines with α subunits to form the binding site for benzodiazepines in GABA_ARs. In addition, a higher level of β3 mRNA was detected. Among the different GABA_AR alpha subunits, higher expression of α5 and unexpectedly lower expression of α1 makes for an uncommon neuronal subtype distribution in comparison to the human adult brain. It is estimated that α5 subunits are found in less than 5% of all the GABA_AR in brain while α1 subunits are found in nearly 60% (Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000). Furthermore, we observed

relatively lower, but similar mRNA levels, for $\alpha 3$ and $\alpha 4$ subunits and even lower expression of $\alpha 2$ and $\alpha 6$ subunits.

Discussion

The results demonstrate that iCell Neurons are an excellent alternative to isolated neurons to measure electrophysiological efficacy of small bioactive ligands on mixed population of human GABA_ARs. iCell Neurons are compatible with various automated patch clamp technologies including the IonFlux microfluidic system and demonstrate high success rates despite their morphology. GABA_AR expression in iCell Neurons has been compared to human brain at various stages of neurodevelopment (Dage, et al., 2014). Interestingly, lower expression of $\alpha 1$ and $\gamma 2$ was observed in human neonatal brain in comparison to adult. In addition, $\beta 3$, $\alpha 4$, $\alpha 5$, $\alpha 2$, and $\alpha 3$ subunits were expressed at higher levels during the first twelve months of post-natal development, but less in the adult brain. Our results are consistent with the observation that iCell Neurons, when cultured for 10 days, are more similar to the GABA_AR expression profile of neonatal than adult human brain. The use of primary neurons would provide more representative GABA_AR patterns but is likely to have disadvantages such as inconsistency of protein expression and viability in culture.

The characterization of α -subtype selectively of new GABA_AR modulators is an essential part of drug discovery. Usually, low efficacy towards the $\alpha 1\beta 3\gamma 2$ GABA_AR is preferred in anxiolytic agents, which would avoid unwanted sedation and tolerance mediated by this receptor. Efficacy characterization involves electrophysiological measurements using recombinant GABA_A receptors expressed in cells with low GABAergic background (Chen, et al., 2012). Using this methodology, many subtype-selective GABA_AR ligands have been developed, like HZ166 and CW-04-020. However, iCell Neurons resulted in ligand affinities and efficacies representing an aggregate electrophysiological response to a compilation of GABA_ARs. The response in turn depended on the receptor subtype-selectivity of the modulator and cellular expression of GABA_ARs. For instance, HZ166 is selective towards the $\alpha 2/\alpha 3/\alpha 5$ GABA_AR subtypes with low affinity for the $\alpha 1$ subunit. In contrast, diazepam causes significant activation of the $\alpha 1\beta 3\gamma 2$ GABA_AR in addition to GABA_ARs bearing $\alpha 2/\alpha 3/\alpha 5$ subunits. With iCell Neurons, we observed affinities of 0.43 μ M for diazepam and 1.56 μ M for HZ166, and potentiation of 590% and 810%, respectively. Thus, the α -subtype-selectivity of GABAergic compounds evaluated herein exhibited different electrophysiological response in iCell Neurons. Furthermore, CW-04-020 has been reported as a $\alpha 6$ -selective positive modulator with very little efficacy for other GABA_ARs subunits. A smaller overall efficacy (480%) was observed in iCell Neurons, due to low expression of $\alpha 6$. The GABA_A receptor subtype distribution in iCell Neurons may be of particular interest in the study of compounds that target $\alpha 5$ containing GABA_A receptors. In humans, $\alpha 5$ has been identified as a susceptibility marker for schizophrenia (Maldonado-Aviles, et al., 2009) and depression (Kato, 2007). This receptor subtype appears to be regulated substantially by stress hormones and expression changes are often associated with stress-related disorders (Sequeira, et al., 2009) and traumatic brain injury (Loup, Wieser, Yonekawa, Aguzzi, & Fritschy, 2000). $\alpha 5$ has also been implicated in learning and memory impediments; thus pointing to selective inhibitors of $\alpha 5$ -containing receptors as cognitive enhancers in Alzheimer's disease patients.

In conclusion, patch clamp studies using recombinantly expressed GABA_AR give valuable information about the effects of ligands at specific GABA_ARs, however ignores the complexity that arises when multiple subtypes are expressed on a single cell. Screening compounds in parallel with human-induced pluripotent stem cell neurons provides a valuable and unique perspective on the effects of new compounds. Due to the elevated expression of $\alpha 5$, it is expected that active compounds have high efficacy toward $\alpha 5\beta 3\gamma 2$ GABA_ARs. iCell Neurons are adaptable to automated patch clamp and can provide a screening tool to determine the total efficacy and potency in human neurons. In light of the GABA_AR expression pattern in iCell Neurons, they may offer an excellent representation of relevant GABA_A receptors to test pharmaceuticals targeting depression, cognitive deficiencies, and severe brain injury. These assays may better model human responses as compared to animal models where the expression levels of GABA_A receptor subunits and their spatial distribution (as in rodents) do not necessarily correspond with the human brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank Cellular Dynamics International, a Fujifilm Company for providing the iCell Neurons utilized in this study. This work was supported by the University of Wisconsin-Milwaukee, the National Institutes of Health R03DA031090, R01NS076517, R01HL118561, R01MH096463 the University of Wisconsin Milwaukee Research Foundation (Catalyst grant), the Lynde and Harry Bradley Foundation, the Richard and Ethel Herzfeld Foundation.

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Figure 1.

Seal resistance of patched clamped iCell Neurons. Twenty individual cells are patched clamped in a trap channel. Two trap channels are part of one microfluidic ensemble and eight ensembles are arranged in a 96 well IonFlux plate.

Figure 2.

GABA-induced current responses of iCell Neurons measured with the IonFlux instrument.

A) Current recordings of different concentrations of GABA applied for 3 seconds using a trap channel that has twenty patched clamped iCell Neurons; B) Concentration-dependent current response curve of GABA using trap channels that have twenty patched clamped iCell Neurons (N = 16); C) Current recordings of different concentrations of GABA applied for 3 seconds using a single cell recording with a trap channel that has one patched clamped iCell Neuron; D) Current recordings of 100 nM GABA (EC_3) applied repeatedly for 3 seconds using a trap channel that has twenty patched clamped iCell Neurons.

Figure 3.

Current responses of twenty patched clamped iCell Neurons from a trap channel in an IonFlux 16 plate in the presence of positive allosteric GABA_AR modulators and GABA. A) Current recordings of iCell Neurons in the presence of different concentrations of diazepam and 100 nM GABA applied for 3 seconds; B) Concentration-dependent current response curve of iCell Neurons in the presence of diazepam and 100 nM GABA ($N = 20$); C) Current recordings of iCell Neurons in the presence of different concentrations of HZ-166 and 100 nM GABA applied for 3 seconds; D) Concentration-dependent current response curve of iCell Neurons in the presence of HZ-166 and 100 nM GABA ($N = 20$); E) Current recordings of iCell Neurons in the presence of different concentrations of CW-04-020 and

100 nM GABA applied for 3 seconds; F) Concentration-dependent current response curve of iCell Neurons in the presence CW-04-020.

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Figure 4.
Quantification of GABA_AR subunit mRNA using qRT-PCR in iCell Neurons after 10 days of culture.