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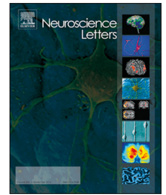
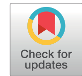


Research article

Plasminogen binding inhibitors demonstrate unwanted activities on $GABA_A$ and glycine receptors in human iPSC derived neurons



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ABSTRACT

Plasminogen binding inhibitors (PBIs) reduce the risk of bleeding in hemorrhagic conditions. However, generic PBIs are also associated with an increased risk of seizures, an adverse effect linked to unwanted activities towards inhibitory neuronal receptors. Development of novel PBIs serve to remove compounds with such properties, but progress is limited by a lack of higher throughput methods with human translatability. Herein we apply human induced pluripotent stem cell (hiPSC) derived neurons in combination with dynamic mass redistribution (DMR) technology to demonstrate robust and reproducible modulation of both $GABA_A$ and glycine receptors. These cells respond to GABA (EC_{50} 0.33 ± 0.18 μM), glycine (EC_{50} 11.0 ± 3.7 μM) and additional ligands in line with previous reports from patch clamp technologies. Additionally, we identify and characterize a competitive antagonistic behavior of the prototype inhibitor and drug tranexamic acid (TXA). Finally, we demonstrate proof of concept for effective counter-screening of lead series compounds towards unwanted $GABA_A$ receptor activities. No activity was observed for a previously identified PBI candidate drug, AZD6564, whereas a discontinued analog, AZ13267257, could be characterized as a potent $GABA_A$ receptor agonist.

1. Introduction

The use of drugs in disease is a delicate balance between therapeutic benefit and risk to the patient. Potential adverse effects must be addressed early in drug discovery efforts to avoid late and costly clinical development failures. Consequently, the initial identification of hit molecules through compound screening is followed by lead series generation, where the chemistry is optimized towards increased efficacy on the desired target protein. In this process the effective removal of undesirable activities is as important, such that high-quality lead series with limited off-target effects are progressed.

The generic antifibrinolytic drug tranexamic acid (TXA) inhibits the protein-protein interaction between plasminogen and fibrin [9]. TXA is frequently used to reduce bleeding in various hemorrhagic conditions, such as during surgical procedures in the heart [19]. However, treatment with TXA is associated with an increased incidence of seizures [15,19]. These adverse events are hypothesized to be due to the competitive antagonist properties of TXA towards the inhibitory neuronal receptors for $GABA_A$ and, possibly, glycine [7,12,15]. A recent search for novel PBIs identified 4-PIOL with improved potency for the primary target, but this compound still shows insufficient selectivity towards the inhibitory $GABA_A$ receptor [3]. In this effort the selectivity against the $GABA_A$ receptor was measured using an animal derived low-throughput membrane binding assay. This counter-screening assay was accurate, but labor intensive, and is thus not considered suitable for assessments across larger compound sets. In addition, this assay provides no information on agonist or antagonist mode of action. After similar internal assay experiences during the identification of candidate drug AZD6564 [4], we aimed to derive a more efficient and informative screening assay for the $GABA_A$ receptor, a very important CNS target both for clinical efficacy and toxicology. In short, the aim was to establish an assay which allows for the identification of activity towards inhibitory neuron receptors $GABA_A$ and glycine in a high-throughput screening setting.

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A prerequisite for these activities was to simultaneously improve translatability through a move from animal-derived cells towards more primary cell types of human origin. Human induced pluripotent stem cells (hiPSC) provide large scale availability of human cells and can potentially be used to address the very limited accessibility of human primary neurons. Human iPSC derived neurons express endogenous levels of inhibitory neuronal receptors [5,21,22] and have been shown to recapitulate important electrophysiological features of neurons using calcium imaging [5], multi-electrode systems (MEA) [1] and patch clamping [8,22]. However, both manual and automated patch clamping [6] are challenging to perform in a screen setting, while MEA is associated with significant analytical and computational challenges [17]. Additionally, neither method is suitable and sufficiently cost effective to allow for high-throughput screens in their current formats. Instead we turned our interest to label free dynamic mass redistribution (DMR) technology, which measures the integrative phenotypic response originating from multiple signaling events in the cells [11]. The technology allows for the use of high density microplates with low cell numbers and short turn-around time making it a suitable candidate for our purpose.

In this study we develop and validate the use of human iPSC derived neurons for measurements of endogenous receptor modulation using high-throughput DMR technology. We show that the inhibitory $GABA_A$ and glycine receptors can be robustly modulated with natural ligands. Moreover, we can detect agonist selectivity between receptor populations comprised of different subunit compositions [18], allosteric modulator potentiation [10,22], and taurine dual receptor agonism [2], thus providing confidence in assay applicability for mechanistic studies. We further demonstrate that our method can detect previously reported off-target activity of TXA against the $GABA_A$ and glycine receptors. Additionally, we demonstrate proof of concept for the evaluation of compounds from a lead series of PBIs for $GABA_A$ receptor with improved selectivity profile. Our study demonstrates the direct applicability and impact of using label free high-throughput technology with human iPSC neurons in drug discovery efforts.

2. Materials and methods

2.1. Modulators

GABA was from Tocris (0344), glycine (50046) and strychnine (85920) were from Fluka, while taurine (T8691), β-alanine (A7752), gabazine (S106), bicuculline (14340), caffeine (C0750), diazepam (D0899), TXA (857653) and picrotoxin (P1675) were all from Sigma-Aldrich.

2.2. Cell culture

Human iPSC neurons (iCell® Neurons, Cellular Dynamics) were thawed and maintained according to the supplier's recommendations. The cells were plated at a density of 17.000 cells per well in 384 well microplates with optical sensors (Corning 5040). The plates had first been pre-coated for 2 h at 37 °C with a base layer of poly-L-ornithin (Sigma-Aldrich), prepared as a 2 µg/ml solution in phosphate buffered saline (PBS, Gibco). After 2 washes with PBS a second coat consisting of 3.3 µg/ml laminin (Sigma-Aldrich) was applied overnight at 37 °C. The seeded cells were cultured in iCell® Neurons Maintenance Medium (Cellular Dynamics, NRM-100-121001) in 5% CO_2 and 37 °C for five days before the experiments were performed. The cell media was changed two days post thaw. The cells used for the reported experiments originated from five cryopreserved vials from the same batch.

2.3. Immunocytochemistry

Cells were fixated in 4% formaldehyde (Sigma-Aldrich) for 15 min at room temperature after five days in culture. The fixated cells were washed in PBS and incubated for 1 h at room temperature with blocking and permeabilization buffer consisting of 10% goat serum and 0.1% Triton X-100 in PBS (all from Invitrogen). The primary antibodies, mouse anti-Tuj1 (1:500, R&D Systems, MAB1195), rabbit anti-GFAP (1:1500, Millipore, AB5804) and chicken anti-MAP2 (1:200, Abcam, AB5392) were diluted in antibody buffer consisting of 4% goat serum and 0.01% Triton X-100 in PBS (all from Invitrogen) and incubated with cells at 4 °C overnight. Cells were washed in PBS prior to application of secondary antibodies Alexa Fluor 488 goat anti-mouse (1:600; Invitrogen), Alexa Fluor 594 goat anti-rabbit (1:600; Invitrogen), and Alexa Fluor 568 goat anti-chicken (1:600; Invitrogen) in antibody buffer and incubation with cells for 45 min at room temperature. Following a wash in PBS a solution of DAPI (1:2000; Invitrogen) in antibody buffer was added for nuclei staining. Images were captured using an ImageXpress wide field microscope and downstream image analysis was performed in the MetaXpress software (both from Molecular Devices, Sunnyvale, CA). Four separate wells having 9 image sites each (n = 28) were analyzed for GFAP and TUBB3 percentage.

2.4. The DMR label-free assay

The Corning® Epic® technology measures a change in light refraction from the optical sensors at the bottom of the plates upon stimulation of the cells with various agents. Mass redistribution occurs across cell populations as a response to *e.g.* receptor activation and this can be followed as a small shift in wavelength of the refracted light. Five days after thawing and plating the hiPSC neurons have matured to support relevant experiments (supplier's verifications). At this point the cells were washed with Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES at pH 7.4 (both from Invitrogen) using an Elx405 cell washer (BioTek). This was followed by a pre-equilibration period for 90 min in the Epic instrument before compound challenge and readout. A base line reading was taken just before the addition of compounds, which was performed using a Cybi™-Well vario 384/40 liquid handler (CyBio). The compounds had first been diluted in HBSS with 20 mM HEPES at pH 7.4 and were dispensed as 4 x solutions to compensate for the dilution in the microplate. Measurements in the Epic instrument were taken once per minute after compound addition for a period of 45 min. The DMR experiments were run in a minimum of n = 3 replicates across two plates or more. For validation of assay robustness over time the stimulations with GABA and β-alanine were run at n = 4-5 across two time points.

2.5. Data analysis and statistics

Analysis of concentration response curves (CRCs) to yield EC_{50}/IC_{50} values was performed using the non-linear regression log (agonist or antagonist) vs response (three parameters) function within GraphPad Prism 7. This analysis was based on the use of the maximum value obtained during the kinetic reading, occurring approximately 3–6 min after the addition of compounds.

3. Results

3.1. Modulation of the $GABA_A$ receptor

In changing from a low-throughput rodent-derived cell model to a human based high-throughput assay for assessment of $GABA_A$ receptor modulation we wanted to assess the use of hiPSC neurons combined with DMR technology (Fig. 1A) [11]. The approach comes with the added benefit of mode of action capability for natural ligands as well as other modulators. Human iPSC derived neuronal cultures display no significant expression of glial fibrillary acidic protein (GFAP) ($< 1\%$, n = 28), but a homogenous β3-tubulin (TUBB3) ($> 95 \pm 0.7\%$, n = 28) (Fig. 1B) and microtubule-associated protein 2 (MAP2) expression (Fig. 1C). Protein expression profile confirm a very

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