Evaluating the Effects of the Muscarinic Receptor Agonist Pilocarpine on Neural Network Activity Using Three Cell Types on Different Days *in vitro* on a Microelectrode Array Platform

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Abstract

Pilocarpine is a muscarinic receptor agonist used for a rat model of epilepsy. It induces status epilepticus at a relatively high dose (380 mg/kg) within 10-30 minutes and after a prolonged duration of status epilepticus, brain injury and neuronal loss occur, producing an epileptic phenotype. We tested pilocarpine in an in vitro model for seizure liability using a microelectrode array platform in three different neuronal cell types; cryopreserved rat cortical neurons, cryopreserved rat hippocampal neurons and a co-culture of human iPSC-derived glutamatergic neurons with astrocytes. We determined that this drug, up to concentrations of 400 µM, did not produce seizurogenic-like responses in 14 DIV rat cortical neurons or 14 DIV rat hippocampal neurons. In the 14 DIV GlutaNeuron/Astrocyte co-culture, however, there was a robust response at 31.6 µM, with decreasing intensities at lower concentrations in a dose response manner. At 31.6 µM, pilocarpine caused up to 2-fold decreases in firing rates, 3-fold increases in percent isolated spikes (spikes occurring outside of bursts) and 5 to 10-fold increases in median/mean ISI and median ISI (indicators of burst structure deterioration) in the 14 DIV GlutaNeuron/Astrocyte co-culture. In subsequent experiments, we retested pilocarpine in the rat cortical neurons and hippocampal neurons at 21 DIV, which produced a much more robust response. The changes in the rat cortical neurons included 2-fold decreases in the number of spikes in bursts and 2-fold increases in the median ISI, both responses indicating a deterioration in burst organization. We also observed a breakdown in network synchrony. This pattern was consistent with the iPSC neuron response observed at 14 DIV. Alternatively, the 21 DIV rat hippocampal neurons responded with an increase in regularity characterized by changes in endpoints such as 3-fold increases in the number of spikes in bursts, 3-fold increases in burst duration and 2-fold decreases in the MAD burst spike number, which indicates an increase spike train regularity. When changes in muscarinic receptor expression over time were measured, expression changes in maturing cells were the cause of the significant differences. In conclusion, understanding the maturity and response patterns of different neuronal cell types is important for determining whether a model is correct for identifying liabilities associated with specific receptors.

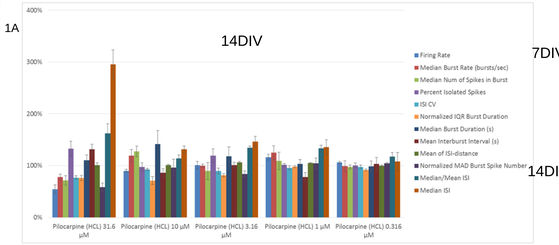
Neurons

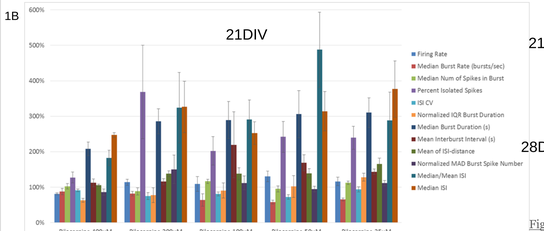
- Cryopreserved Rat Cortical Neurons (QBM Cell Science)
- Cryopreserved Rat Hippocampal Neurons (QBM Cell Science)
- iCell GlutaNeurons: iPS cell-derived human cortical neurons consisting primarily of 90% glutamatergic (excitatory) neurons (Fujifilm Cellular Dynamics, Inc.)
- iCell Astrocytes: iPS cell-derived human astrocytes (>95% pure astrocytes) (Fujifilm Cellular Dynamics, Inc.)

Methods

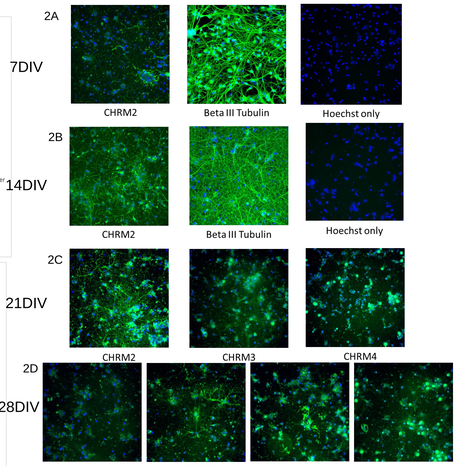
- Microelectrode array evaluation
  - 48-well MEA plates were pre-coated with a 0.1% PEI solution and allowed to dry overnight. One hour before plating cells, the plates were treated with a laminin solution by dispensing a 10 or 15µL dot (depending on cell type) directly over the electrode grid and incubating at 37°C.
  - Cryopreserved rat cortical neurons (QBM Cell Science), hippocampal neurons (QBM Cell Science) and CDI's iCell GlutaNeurons and Astrocytes were rapidly thawed and slowly diluted with the appropriate medium.
  - After a gentle centrifugation step, the cells were resuspended at the appropriate density with the appropriate medium.
  - The laminin droplet was aspirated and replaced with the same volume of the cell suspension at the appropriate density.
  - The cells were incubated, humidified at 37°C in 5% CO2 for 2 hours before medium was added to the wells.
  - Cells were maintained for 14-28 days by changing 50- 60% medium 3 times a week.
  - Microelectrode Array Recordings were acquired on the Axion BioSystems' Maestro immediately before compound treatment (baseline) and 1 hour post treatment.
  - Custom MATLAB scripts were used to analyze the spike trains for data generated for all of the cell types.
- Cell staining and imaging
  - 384-well clear bottom tissue culture plates were treated with a laminin solution one hour to plating cells.
  - Cryopreserved rat cortical neurons (QBM Cell Science), hippocampal neurons (QBM Cell Science) and CDI's iCell GlutaNeurons and Astrocytes were rapidly thawed and slowly diluted with the appropriate medium.
  - After a gentle centrifugation step, the cells were resuspended and plated at 10,000 cells per well.
    - The iCell GlutaNeurons and Astrocytes were plated as a co-culture with 85% being GlutaNeurons
  - Cells were fixed at DIV 7, 14, 21 or 28.
  - After a permeabilization and blocking step, the cells were stained with the following:
    1. Muscarinic acetylcholine receptor M1 (CHRM1) and Hoechst
    2. Muscarinic acetylcholine receptor M2 (CHRM2) and Hoechst
    3. Muscarinic acetylcholine receptor M3 (CHRM3) and Hoechst
    4. Muscarinic acetylcholine receptor M4 (CHRM4) and Hoechst
    5. Beta III Tubulin and Hoechst
    6. Hoechst only
  - Cells were imaged with a CellInsight CX7 High-Content Screening (HCS) Platform (ThermoFisher Scientific).

Results: Cryopreserved Rat Cortical Neurons



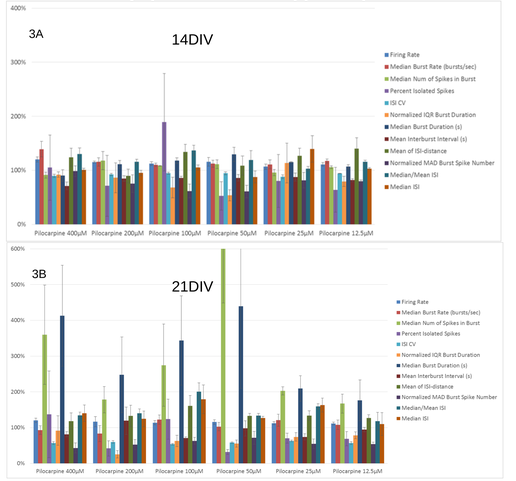


Figures 1A and B. Initial testing with pilocarpine in cryopreserved rat cortical neurons at 14 DIV and up to concentrations of 100µM did not have a phenotypic seizurogenic response in this assay. 1A is a graph of pilocarpine dose response with a top concentration of 31.6µM. 1B. Follow-up testing with pilocarpine in cryopreserved rat cortical neurons at 21 DIV and up to concentrations of 400µM. The effects of pilocarpine at all concentrations tested shows a substantial and deleterious effect on burst organization and a decline in synchrony.

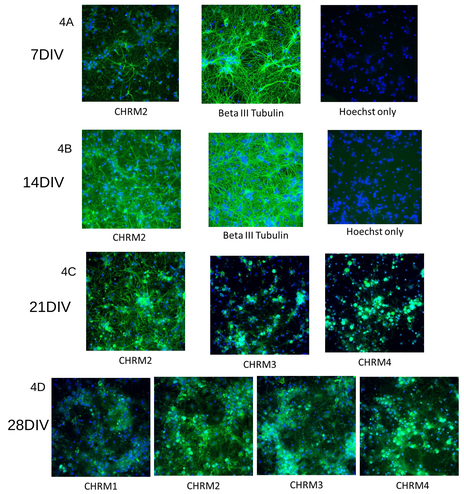


Figures 2A, B, C and D. High content imaging of rat cortical neurons on 7, 14, 21 and 28 DIV. The neurons are stained for imaging with Hoechst (all cells) and then various antibodies for detecting Muscarinic acetylcholine receptors M1 , M2, M3, M4 and Beta III Tubulin. 2A and 2B. Images for M2, Beta III tubulin and Hoechst are represented. M1, M3 and M4 were not detected when stained at DIV 7 and 14. 2C. On 21 DIV, M2, M3 and M4 are detected but there is no detectable M1 found. 2D. On 28 DIV, M1 is now slightly detectable, while M2, M3 and M4 detection levels continue to be more robust.

Results: Cryopreserved Rat Hippocampal Neurons

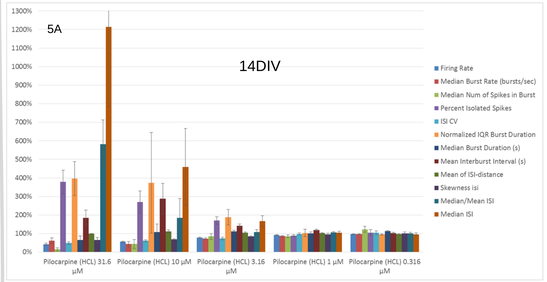


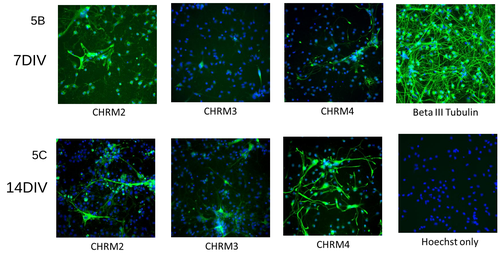
Figures 3A and B. Pilocarpine tested in cryopreserved rat hippocampal neurons at 14 and 21 DIV and up to concentrations of 400µM. The effects of pilocarpine on the rat hippocampal neurons at 14 DIV (3A) was nominal at all concentrations tested. The analysis of the spike trains with customized MATLAB scripts pick up some minor but not significant fluctuations. Pilocarpine tested in cryopreserved rat hippocampal neurons at 21 DIV (3B) caused substantial effects at all concentrations. The pattern of effects on burst characteristics, which changes significantly from the 14 DIV phenotype, becomes much more pronounced. This is observed at all concentrations but does seem to attenuate slightly at the lower concentrations.



Figures 4A, B, C and D. High content imaging of rat hippocampal neurons on 7, 14, 21 and 28 DIV. The neurons are stained for imaging with Hoechst (all cells) and then various antibodies for detecting Muscarinic acetylcholine receptors M1 , M2, M3, M4 and Beta III Tubulin. 4A and 4B. Images for M2, Beta III tubulin and Hoechst are represented. M1, M3 and M4 were not detected when stained at DIV 7 and 14. 4C. On 21 DIV, M2, M3 and M4 are detected but there is no detectable M1 found. 4D. On 28 DIV, M1 is now slightly detectable, while M2, M3 and M4 detection levels are more robust.

Results: iCell GlutaNeurons and Astrocytes Co-culture





Figures 5A, B and C. Pilocarpine tested in FCDI iCell GlutaNeurons and Astrocytes co-culture at 14 DIV up to 31.6µM (5A). The response to pilocarpine in the GlutaNeurons/Astrocytes co-culture was more robust than in the rat cortical and hippocampal neurons at 14 DIV. Firing rates are substantially reduced, burst organization is almost completely disrupted and network synchrony is deleteriously impacted. This effect is observed to be in a dose response manner. 5B and C. High content imaging of the GlutaNeurons/ Astrocytes co-culture on 7 and 14 DIV. The neurons are stained for imaging with Hoechst (all cells) and then various antibodies for detecting Muscarinic acetylcholine receptors M1 , M2, M3, M4 and Beta III Tubulin. At 7 DIV, M2 is easily detected with less significant evidence of M3 and M4. By 14 DIV, M2 is still clearly present with greater detection levels evident for M3. M4 is significantly more expressed at 14 DIV when compared to 7 DIV in this co-culture.

Conclusions

- Initial testing of pilocarpine with cryopreserved rat cortical neurons did not produce a significant phenotypic response at any of the concentrations tested (up to 100µM) at 14 DIV
  - Follow-up testing at higher concentrations (up to 400µM) at 14 DIV increased the effects on firing, bursting and synchrony endpoints but still did not produce any significant response.
  - Follow-up testing at 21 DIV significantly increased the effects on firing, bursting and synchrony endpoints
- Initial testing of pilocarpine with cryopreserved rat hippocampal neurons at 14 DIV did not produce a significant phenotypic response at any concentration tested and had little effect on any of the endpoints
  - Follow-up testing at 21 DIV substantially increased the effects on bursting characteristic and synchrony
- Initial testing of pilocarpine with CDI iPSC-derived GlutaNeurons and Astrocytes co-culture (CDI) at 14 DIV demonstrated a substantial effect on firing, bursting and synchrony endpoints in a dose response manner
- To determine whether muscarinic acetylcholine receptor expression levels may be contributing to these differences, we stained specifically for muscarinic acetylcholine receptors M1, M2, M3 and M4 at 7 and 14 DIV in all three cell types evaluated, and at 21 and 28 DIV in the rat neurons.
- Overall, the expression of muscarinic acetylcholine receptors varied for the three cell types
  - The iCell Glutaneurons expressed M2, 3, and 4 at 14 days
  - The rat cortical neurons express M2 on day 14 but didn't express M3 and 4 until day 21.
  - The rat hippocampal neurons express M2 on day 14 but didn't express M3 and 4 until day 21.
- The results suggest the pilocarpine response is mediated by M3 or M4. RT-PCR will be run for confirmation of expression

References

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