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Abstract

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In the early 1940s, organophosphates used in insecticides were discovered to act as chemical nerve agents[1]. The most common nerve agents are: isopropyl methylphosphonofluoridate (sarin), ethyl dimethylphosphoramidocyanidate (tabun), and S-2-diisopropylaminoethyl O-ethyl methylphosphonothioate (VX). These nerve agents are responsible for causing acetylcholinesterase (AChE) inhibition which leads to acetylcholine gathering in the synapses[2]. This accumulation causes hyperactivity in the nervous system which causes seizures, bradycardia, respiratory paralysis, and eventually death. For this study, the effects of a sarin chemical surrogate, 4-nitrophenyl isopropyl methylphosphonate (NIMP), was evaluated along with the pre-treatment, pyridostigmine bromide (PB) on 3D cultures of human neurons placed on the novel multi-electrode array (MEA). Neuronal spiking and bursting activity was monitored throughout the experiment and levels of cytotoxicity were determined using the lactate dehydrogenase (LDH) assay. In addition, in-vivo analysis was performed for comparison.

[1] Synthesis and In Vitro and In Vivo Inhibition Potencies of Highly Relevant Nerve Agent Surrogates
[2] Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents

Background

The production of organophosphates for potential insecticide treatments led to the inadvertent discovery of chemical nerve agents. During World War II, nerve agents were produced in mass quantities and eventually kept as stockpiles. The most common nerve agents that have been created and studied are: isopropyl methylphosphonofluoridate (sarin), ethyl dimethylphosphoramidocyanidate (tabun), and S-2-diisopropylaminoethyl O-ethyl methylphosphonothioate (VX). The mechanism behind these nerve agents is to phosphorylate the serine found in the active site of the enzyme acetylcholinesterase (AChE) which will prevent the enzyme's hydrolytic action[1]. With the inhibition of AChE, the neurotransmitter acetylcholine accumulates in the synapses leading to hypercholinergic stimulation in the central and peripheral nervous system[3].

Present therapeutics, like 2-PAM or Pyridostigmine bromide (PB), are drugs capable of reactivating the AChE in the peripheral nervous system, restoring the enzyme's function[4]. However, these therapeutics are unable to cross the blood-brain barrier, which fails to reactivate the inhibited AChE enzymes in the central nervous system. In efforts to develop novel treatments that could potentially cross the blood-brain barrier, chemical surrogates are often used in place of nerve agents due to the high risk involved. A common chemical surrogate of the nerve agent sarin is 4-nitrophenyl isopropyl methylphosphonate (NIMP). NIMP inactivates AChE in a similar manner as sarin by using the same chemical moieties as the nerve agent[22].

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[1] Synthesis and In Vitro and In Vivo Inhibition Potencies of Highly Relevant Nerve Agent Surrogates
[3] Testing of novel brain-penetrating oxime reactivators of acetylcholinesterase inhibited by nerve agent surrogates
[4] Efficacy of novel phenoxyalkyl pyridinium oximes as brain-penetrating reactivators of cholinesterase inhibited by surrogates of sarin and VX

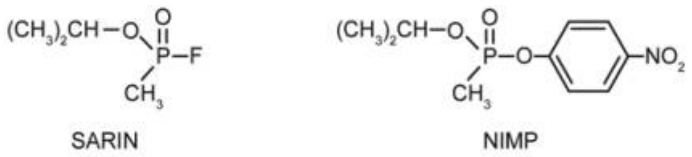

Figure 1: Chemical Structure of Sarin and its surrogate, NIMP[1].

The goal of this project is to characterize the 3D Brain-on-a-chip system and to identify how NIMP alters neuronal networks. We have created a novel 3D system which allows us to use cultured tissue to stimulate physiological conditions since it is unethical to test humans directly in an in-vivo manner. We are evaluating human and rat neuronal in-vitro systems. With this study, we hope to understand how neuronal cells are affected when subjected to NIMP. This data will aid in informing potential treatments to counteract NIMP's toxic effects.

Methods

Prior to determining the effects of NIMP on the neuronal networks, characterization of the probes and neurons were examined.

Probe Angle Analysis: Images of the probes on the MEA devices (see Figure 2) taken from the goniometer, confocal, and bright field microscopes before, during, and after actuation were analyzed using ImageJ to calculate probe angles.

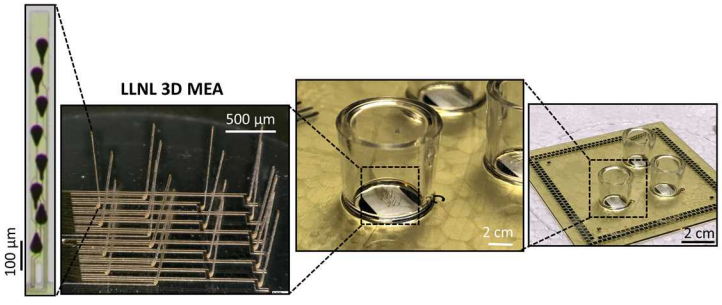

Figure 2: LLNL 3D device with actuated probes.

Cell culture and Electrophysiology Analysis: Human iPSC-derived neurons and astrocytes (Neucyte) were cultured in 3 mg/ml collagen gels on the 3D device. Spiking and bursting activity features were analyzed over a period of 35 days using OpenBridge, Plexon, the multi-channel recording system and a custom analysis package.

Immunocytochemistry Staining: Human neurons in collagen gels were stained with primary and secondary antibodies tagged with fluorophores. The primary antibodies were: Tuj1, Map2, and Gad67.

LDH Assay: Co-cultured human neurons and astrocytes were taken from the MEA devices and the LDH protocol was followed using the ThermoFisher CyQUANT LDH Cytotoxicity Assay. Plates were read at two different absorbances levels of 490nm and 690nm.

Results

Probes angles were measured from the MEA devices to determine if the probes had moved over time. Factors contributing to the changes in angle measurement could be from gel strain placed on the probe or movement during the actuation process. Side view images of the probe angles were calculated through ImageJ which can be seen in Figure 3. The fluorescence represents the probe whose angle was measured.

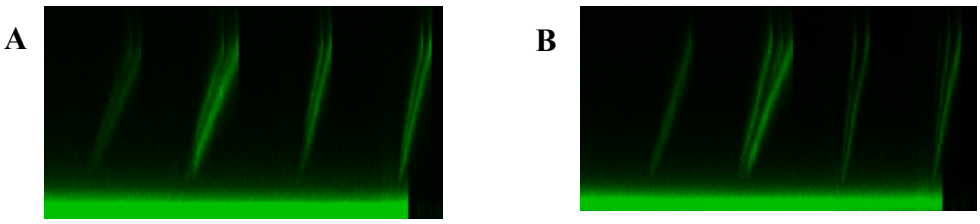

Figure 3: Fluorescent Probes on W17C1–Well A (A) and W17C1–Well C (B) at 20X taken prior to cell seeding.

Looking at Figure 4, consistency between the probe angles was calculated during the 28-day in-vitro experiment. This is important to determine because we are looking for reproducibility between devices, where on the gel are the probes picking up the electrical activity and if we can map out how neurons are forming networks within in the gel.

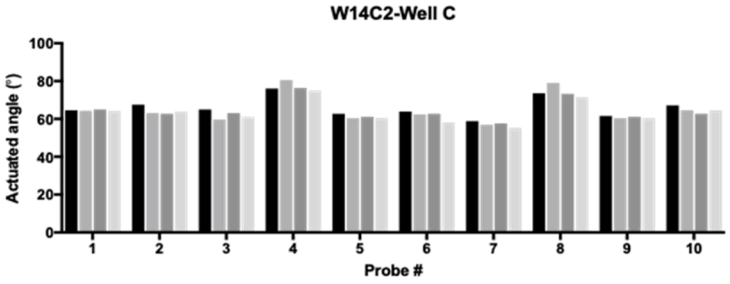

Figure 4: W14C2–Well C demonstrates the consistency in the probe angles seen over 28 days in-vitro (DIV)

These probes can detect how the neurons are able to electrically communicate with each other and in order to determine the spatial orientation of these neurons on the MEA devices, immunocytochemistry staining was performed. The staining gave us a good estimate regarding the cell density and cell types within in the hydrogel. From Figure 5, we can visualize the 3D layer of the hydrogel. The red staining (Tuj1) represents the entire cytoskeleton of the neurons while the yellow staining (MAP2) represents the dendrites and the cell body. With this, we can characterize the subregions of a neuron.

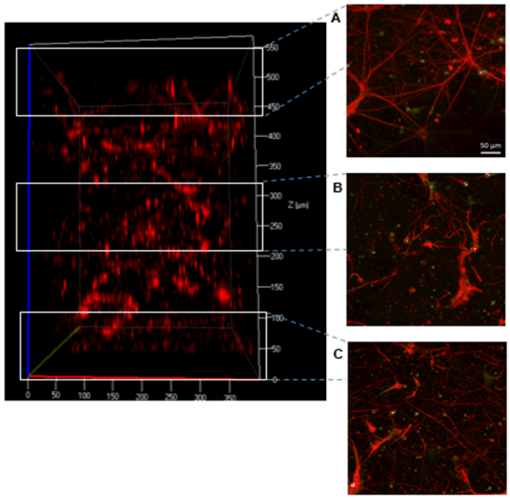

Figure 5: 3D image of human neurons in the XYZ plane. **A.** XY plane image of neurons in the top 20% of the XYZ plane. **B.** XY plane image of neurons in the middle 20% of the XYZ plane. **C.** XY plane image of neurons in the bottom 20% of the XYZ plane. The red stain (Tuj1) represents the entire cytoskeleton while the green stain (MAP2) represents the cell body and dendrites of the neuron. The co-expression is represented by the yellow stain which includes the cell body and dendrites of the neurons.

To determine if pre-treatment of pyridostigmine bromide was effective at blocking the effects against NIMP, a challenge experiment was performed. The three wells in the MEA device each contained a different set of conditions. Well A contained a set of human-iPSC derived neurons, well B contained neurons exposed to NIMP, and well C contained neurons pre-treated with PB prior to NIMP exposure. What we expected to see was the NIMP-treated cells in well B would have a decrease in neuronal activity over time while the pre-treated cells of well C would maintain their activity against NIMP as seen in the baseline activity of control cells. Figure 6 shows a visual diagram of the experiment.

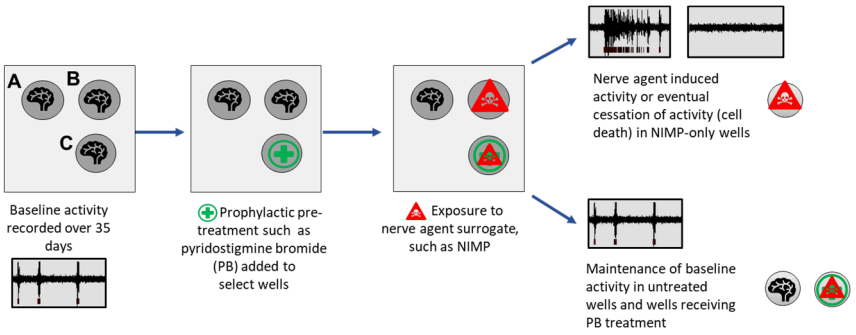

Figure 6: Schematic of PB + NIMP experiment on the MEA device and the activity that should be seen

Baseline activity of human-iPSC derived neurons was monitored over a 35-day period in 30 minutes interval where the spiking and bursting activity was analyzed for all three well conditions. Figure 7 represents an example of the baseline activity seen on the human-iPSC derived neurons that was prepared in rat neural basal media.

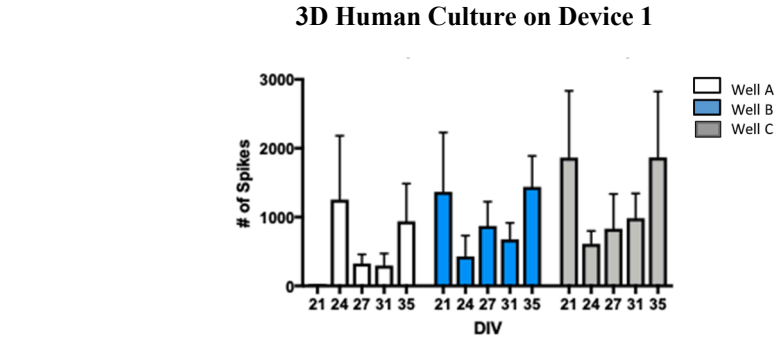

Figure 7: 30-minute baseline activity of human-iPSC derived neurons prepared in rate neural basal media

Electrophysiology analysis was performed during the challenge experiment in 30-minute intervals as well to mimic the baseline activity. Looking at Figure 8, we can see that the spiking and bursting activity of the cells treated with NIMP increased at 24 hours while the cells treated with PB decreased at 24 hours. This preliminary data suggests that there might be neuronal dysfunction.

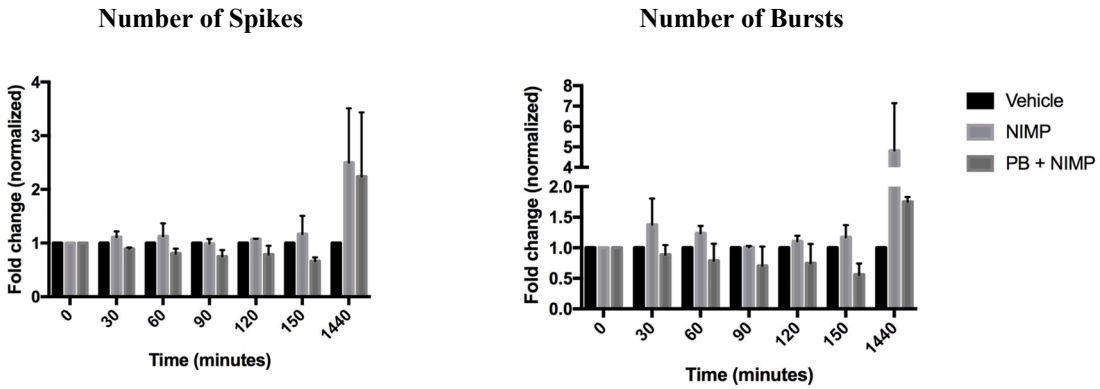

Figure 8: Changes to electrophysiology with NIMP and pyridostigmine bromide (PB) exposure. Two features are shown, number of spikes and bursts.

To determine whether NIMP exposure induced cell death, the LDH assay was performed. The LDH assay is a quantitative method that is used to determine the number of dead cells in the culture medium. LDH is an enzyme found in the cytosol of cells and released into the culture medium when the cell's plasma membrane is compromised. In Figure 9, the cells treated with NIMP had more cell death relative to the control. The cells treated with the pre-treatment of PB and then subjected to NIMP had slightly greater cell death relative to the control.

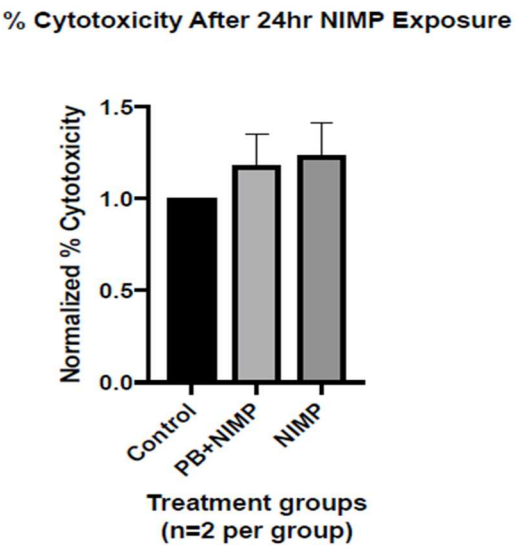

Figure 9: Average amount of cytotoxicity observed in the co-culture for each condition.

Currently, we are comparing our human cell NIMP data to the rodent system. This process also includes using the novel 3D MEA device and comparing the in-vitro system to an in-vivo rodent system (Figure 10). This will allow us to see how the MEA device responds in-vivo. To determine which responses were real, Plexon was used to manually identify action potentials that represented a real signal as shown in Figure 10.

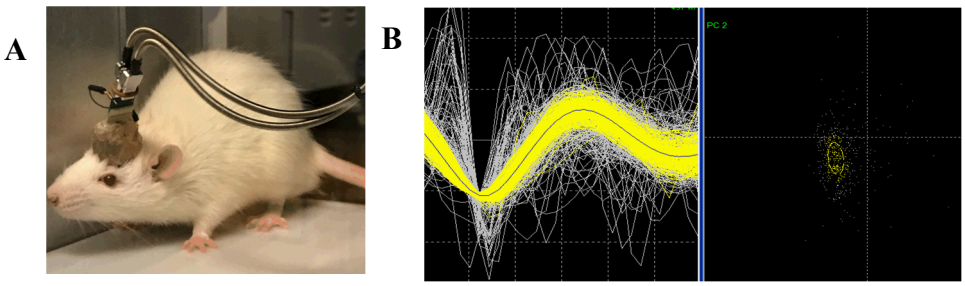

Figure 10: A. Rodent with an implantable MEA device. **B.** Examples of baseline activity identified from the rodent with the implantable MEA device.

Conclusion

In conclusion, the brain-on-a-chip model allowed for further understanding of how neuronal cells behave when subjected to the chemical surrogate, NIMP. The accuracy of probe measurements was determined by the angle of the probes. The angles calculated demonstrated higher consistency of the probes on the MEA devices that were situated at 90 degrees. The immunocytochemistry staining allowed us to identify and visualize subregions of the neuron. For example, the cell body and dendrites (shown in yellow) highly express the protein MAP2, while Tuj1 (red) is expressed in all parts of the neuron. The LDH assay showed a greater level of toxicity to cells treated with NIMP than cells treated with DMSO. Electrophysiology analysis showed differences between groups (PB/NIMP, NIMP) for both number of spikes and bursts over time. The NIMP group showed an increase in spiking and bursting activity at 24 hours. We would have expected a decrease in activity due to more cell death. This may indicate neural dysfunction. In addition, the LDH assay also illustrated the importance of potentially developing a treatment that can counteract the effects of NIMP from the PB+NIMP treated cells. PB offered very little protection against NIMP in our study.

With these findings, we hope to identify potential treatments to counteract the effects of NIMP in future experiments. Regarding the novel MEA devices, they can be applied to a variety of different compounds, including other toxicants and potential therapeutics.

Acknowledgements

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References

1. Meek, E. C., Chambers, H. W., Coban, A., Funck, K. E., Pringle, R. B., Ross, M. K., & Chambers, J. E. (2012). Synthesis and in vitro and in vivo inhibition potencies of highly relevant nerve agent surrogates. Toxicological Sciences, 126(2), 525-533.
2. Albuquerque, E. X., Pereira, E. F., Aracava, Y., Fawcett, W. P., Oliveira, M., Randall, W. R., ... & Adler, M. (2006). Effective countermeasure against poisoning by organophosphorus insecticides and nerve agents. *Proceedings of the National Academy of Sciences*, *103*(35), 13220-13225.
3. Chambers, J. E., Chambers, H. W., Meek, E. C., & Pringle, R. B. (2013). Testing of novel brain-penetrating oxime reactivators of acetylcholinesterase inhibited by nerve agent surrogates. *Chemico-biological interactions*, *203*(1), 135-138.
4. Chambers, J. E., Chambers, H. W., Funck, K. E., Meek, E. C., Pringle, R. B., & Ross, M. K. (2016). Efficacy of novel phenoxyalkyl pyridinium oximes as brain-penetrating reactivators of cholinesterase inhibited by surrogates of sarin and VX. *Chemico-biological interactions*, *259*, 154-159.