Utilizing DREADDs to Transiently Open the Blood Brain Barrier for Drug Delivery

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Utilizing DREADDs to Transiently Open the Blood Brain Barrier for Drug Delivery

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Abstract

The blood-brain barrier (BBB) prevents many therapeutics from reaching and treating the brain tissue. Designer receptors exclusively activated by designer drugs (DREADDs) are a type of chemogenetic tool, currently utilized to study patterns of neural firing in research. This paper aims to repurpose DREADDs for application in endothelial cells of the BBB. A specific DREADD was employed that, when activated by the designer drug clozapine N-oxide (CNO), triggers a cell signaling pathway that results in decreased cyclic adenosine monophosphate (cAMP) levels. This paper hypothesizes that lowered cAMP levels, due to DREADD activation, would increase the permeability of the BBB. To assess how DREADDs affect membrane integrity, a computational model was used to simulate the effects of activated DREADDs on tight junction protein activity levels. To validate the predictions, immunofluorescence (IF) staining of the tight junction protein claudin-5 *in vitro* was performed. To examine how DREADDs impact cell monolayer resistance, electric cell-substrate impedance sensing (ECIS) was conducted to measure resistance *in vitro*. The results suggest that when activated by CNO, the DREADD of interest is capable of modulating cell monolayer resistance, decreasing claudin-5 activity levels as one mechanism of action.

Keywords: blood-brain barrier, designer receptors exclusively activated by designer drugs, claudin-5, cell monolayer resistance

Introduction

The blood-brain barrier (BBB) is a complex network of vasculature that regulates the movement of cells, ions, and molecules between the brain and circulatory system. Accordingly, the BBB protects the brain from pathogens and harmful substances in the blood, largely through the endothelial cells that line the blood vessels of the BBB.[1] Tight junctions seal the interendothelial cleft, a gap between endothelial cells through which molecules may travel, to create continuous blood vessels and limit paracellular diffusion.[2] Due to its filtering of substances, the BBB provides an obstacle to delivering therapeutics to brain tissue, specifically when the drug molecules are larger than 400 Daltons and/or lipophobic.[3] This strict BBB filtering prevents over 98% of small-molecule drugs from entering the brain.[3]

Alzheimer's disease is a prevalent neurological disease where access to the brain is hindered by the BBB. In 2012, 5.5 million Americans and 24 million people worldwide had Alzheimer's disease and in 2021, it was listed as the 7th leading cause of death.[4,5] Alzheimer's disease is the most expensive condition to treat in the United States, generating $321 billion in healthcare costs annually.[6] Two major indicators of Alzheimer's disease are the presence of proteins tau and beta-amyloid. These proteins build up and clump together, surrounding neurons and impeding neuronal function.[7] Current drugs are not able to easily pass through the BBB for AD treatment. To compensate, patients are given extremely high drug doses that can lead

to serious side effects such as brain bleeds[8]. As an example, the drug aducanumab is FDA-approved for Alzheimer's treatment by reducing amyloid plaques in the brain. At high injection doses, there is clear evidence that this drug is able to pass the BBB, but this accompanies a phenomenon known as amyloid-related imaging abnormalities of edema (ARIA-E).[9] A drug delivery platform that allows targeted delivery bypassing the BBB can decrease dose amounts and minimize the risk of serious side effects.

Current nanopharmaceutical methods are also limited. An example is viral vectors, which infect cells with nucleic acids for drug delivery; yet, these vectors have numerous limitations, including safety concerns, high costs, difficulties bypassing the BBB, and require direct administration to the brain.[10] Invasive techniques, such as viral vectors, present risks of brain trauma, surgical complications, and amiss targeting.[11]

To address this challenge, BBB disruption is a method that seeks to transiently create pores in the BBB in order to deliver drugs to the brain tissue.[12] An existing technology that does this utilizes osmotic disruption of the BBB. An osmotic agent draws water out of the BBB and into the lumen of the blood vessels. As water flows out of the blood vessels, vasodilation occurs to compensate for this. The endothelial cells shrink in size as a result, loosening the tight junctions that define the impermeability of the BBB.[13] Major limitations of this platform include the potential for cerebral edema and the non-specificity towards BBB endothelial cells.[14] One technique is focused ultrasound (FUS), which utilizes microbubbles in combination with ultrasound.[15] When the microbubbles pass through the field created by the ultrasound, they begin to oscillate at the same frequency as the ultrasound. This oscillation produces a mechanical force on the walls of the blood vessels, creating transient BBB openings.[16] When considering FUS treatment, there are limitations that patients must accept relating to comfort and treatment compliance. Patients are required to travel to a treatment site for hours of treatment.[17] FUS requires chronic exposure, placing patients under anesthesia.

The aim of this paper is to utilize Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) for an effective drug delivery platform targeting the brain. DREADDs are engineered molecules that contain genes to encode for specific protein receptors. Once the DREADDs are uptaken by and the protein receptors are assembled within the cells, a designer drug can be sent through the bloodstream to bind with the receptors. Due to this coupling, cellular pathways are activated or inhibited, allowing each specific DREADD to induce a specific cellular activity.

This paper assesses the ability of the hM4Di DREADD to temporarily modulate cell monolayer permeability. This paper hypothesizes that when activated by CNO, the hM4Di DREADD would reduce cAMP levels to correspondingly reduce tight junction protein activity and cell monolayer resistance. To test this hypothesis, 3 aims were proposed and executed. First, a computational model was employed to predict the effects of DREADDs on the activity of tight junction proteins. Then, these predictions were tested *in vitro* through IF staining of a tight junction protein. Lastly, ECIS was utilized to examine the impact of DREADDs on cell monolayer resistance *in vitro*.

Results

Computational Model

The Saucerman Lab's computational model, Netflux, was used to simulate tight junction protein activity relating to DREADD activation.[18] A model for endothelial cells, created by members of the Price Lab, was modified to represent endothelial cells transfected with DREADDs. The endothelial cell model was inputted into Netflux. In Figure 1, the activity level of 3 crucial tight junction proteins in DREADD-transfected endothelial cells, in the presence or absence of CNO activation, was simulated.

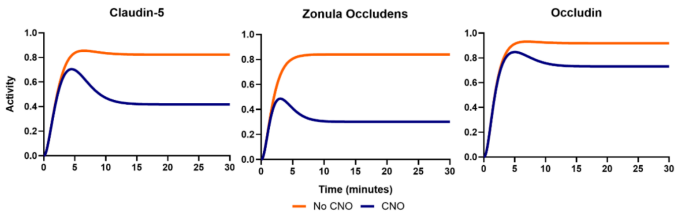

Figure 1. Claudin-5, zonula occludens, and occludin protein activity in DREADD-transfected endothelial cells simulated by Saucerman Lab Netflux computational model. Simulated data represents DREADD-transfected bEnd.3 cells unactivated (orange) and activated (blue) with the designer drug CNO.

Immunofluorescence Staining

Qualitative Fluorescence

To confirm the model's prediction, IF staining was performed to stain for claudin-5. In previous literature, a relationship between cAMP levels and claudin-5 expression levels has been demonstrated in porcine BBB endothelial cells.[19] It was hypothesized that the hM4Di DREADD, which decreases cAMP levels, would also have downstream effects on the claudin-5 levels. Furthermore, it was expected that DREADD-treated and CNO-activated cells would exhibit decreased red fluorescence, relating to reduced claudin-5, compared to the control. Presented in Figure 2, each image is a representative portion of the wells imaged 400 um by 400 um. A significant visual qualitative difference can be observed between the amount of red fluorescence in the DREADD-treated wells versus the control wells. Visually, claudin-5 levels between the experimental groups that received different concentrations of CNO appear equivalent.

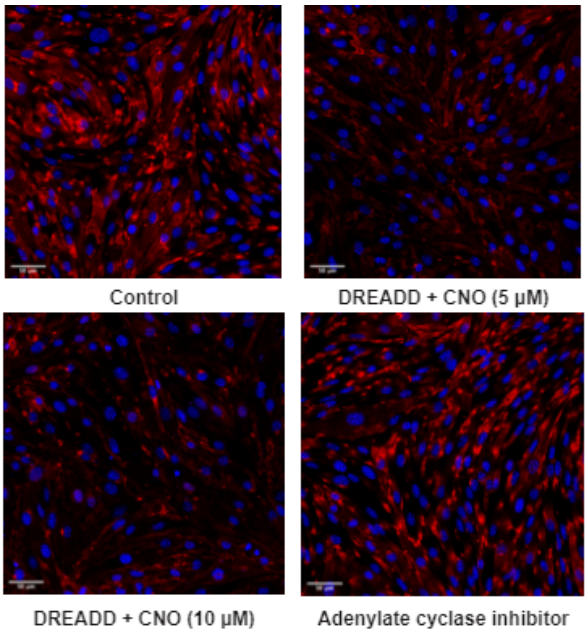

Figure 2. Treatment groups (control, DREADD-transfected and activated with 5/10 μM CNO, and adenylate cyclase inhibitor) stained with claudin-5 (red) and nuclei (blue).

The hM4Di DREADD acts to decrease cAMP levels by inhibiting adenylate cyclase, the enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cAMP.[20] To better understand the mechanism by which the DREADDs could affect permeability and claudin-5 expression, an adenylate cyclase inhibitor drug, SQ22536, was employed.[21] In the bottom right image, cells are treated with this drug. Qualitatively, the amount of claudin-5 in these cells appears comparable to the amount in the control wells. The next section provides a quantitative analysis of the IF images.

Quantitative Fluorescence

Although qualitative fluorescent results suggested a reduction of claudin-5 expression after treatment, quantitative analysis was needed to determine if differences in fluorescence levels were significant. For each well, two images with a confocal microscope were taken to image claudin-5 and DAPI, which stains cell nuclei. The images were taken at 20x objective. Using ImageJ, these two images were overlaid using the "images to stack" function. Then, a 300 by 300 pixel snapshot was isolated, and the number of nuclei was counted. Next, the red channel (in which claudin-5 was imaged) was set to the same minimum and maximum exposure, which allowed for a direct comparison of fluorescence intensities. Finally, the mean gray value was collected for each image.

Once each well had four measurements taken in different locations, the mean gray value was divided by the amount of cells in its respective section, allowing for direct comparison between images. As presented by Table I, Table II, and Figure 3, the mean gray value per cell in the control group is 0.166 +/- 0.014, while the mean gray

Table I: Average mean gray value per cell represented as ± standard deviation.

Group	Mean Gray Value Per Cell ± S.D.
Control	0.1665 ± 0.0143
DREADD and 5 μM CNO	0.1084 ± 0.0139
DREADD and 10 μM CNO	0.0963 ± 0.0136
10 μM Adenylate Cyclase Inhibitor	0.1061 ± 0.0137

Table II: Associated p-values for compared groups. Unpaired t-test was utilized with a 95% confidence interval.

	P- Value
Control versus. DREADD and 5 μM CNO	0.0367
Control versus. DREADD and 10 μM CNO	0.0017
Control versus. 10 μM Adenylate Cyclase Inhibitor	0.0032

value per cell for the DREADD with 5 uM CNO, DREADD with 10 uM CNO and the adenylate cyclase inhibitor is statistically significantly lower by a minimum of 0.058. The p-value between all other treatment groups, excluding the control group, was not statistically significant with a 95% confidence interval. Therefore, there is no statistical difference between the drug-treated cells and CNO-activated DREADD-treated cells, indicating that the mechanism of action is comparable.

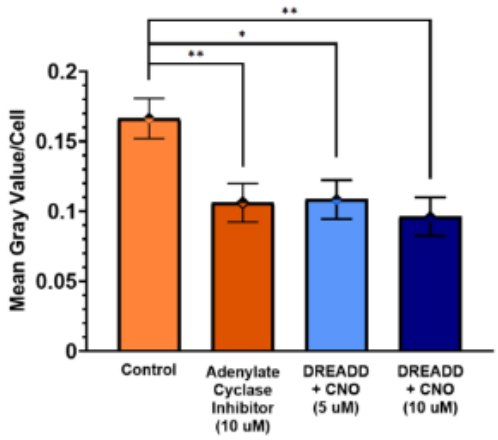

Figure 3. Mean gray value per cell data represented for untreated bEnd.3 cells, adenylate cyclase inhibitor treated cells, and activated DREADD-transfected cells treated with 5 μM and 10 μM CNO. Values shown are mean +/- S.D. with n=3. Unpaired t-test (95% confidence interval). ** p<0.05, ** p<0.01*

Electric Cell-Substrate Impedance

ECIS experiments were conducted to further examine the mechanism by which DREADDs affect permeability. Resistance measurements were collected incrementally every ~ 2 minutes from the time of transfection until four days post-drug application. All treatment groups that were transfected were normalized to the resistance values measured 3 hours prior to removing the plate from the incubator to exclude disruptions generated from removing the plate from the incubator and applying treatments. The variations of these resistance values were accounted for by averaging all resistance values prior to drug application and dividing every treatment group by this averaged value to normalize.

Drug Application

To measure the resistance change between the control cells and cells treated with SQ 22536, the normalized resistance

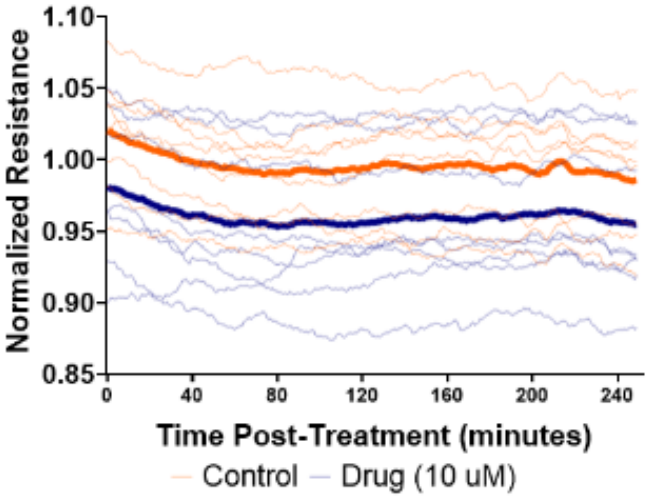

Figure 4. Normalized resistance of treatment groups (untreated bEnd.3 cells and adenylate cyclase inhibitor (10 μM)) post-treatment. Repeated measures two-way ANOVA test (95% confidence interval) yielded a p value of 0.0005.

values of these two groups were compared. Figure 4 reveals the average resistance values for both treatment groups (over the course of 4 hours post-drug application) in the bold lines, along with the 8 replicates to allow for visualization of variation for each group. To determine the statistical significance of the two groups, a repeated measures two-way ANOVA test yielded a significant p-value of 0.0005. In Figure 5, resistance values between 50 and 60 minutes post-treatment were averaged for each group and compared by a non-parametric Mann-Whitney U test, producing a significant p-value of <0.0001. The statistical tests indicate that there is a clear difference between the normalized resistances of the two groups. These results support our hypothesis that the mechanism of action of the hM4Di DREADD (lowered levels of cAMP via inhibition of adenylate cyclase) can modulate resistance (and by extension, permeability) of BBB endothelial cells.

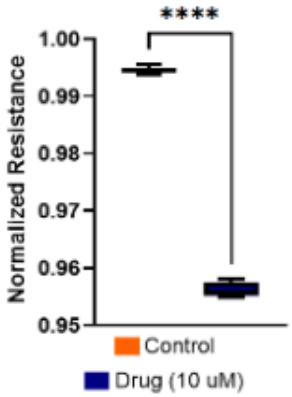

Figure 5. Normalized, averaged resistance of treatment groups (untreated bEnd.3 cells and adenylate cyclase inhibitor (10 μM)) from 50-60 minutes post-treatment. Mann-Whitney test (95% confidence interval).***** p<0.0001*

DREADD Activation

The experimental setup to determine the effect of activated DREADDs on endothelial cell resistance attempted to account for confounding variables. To have the bEnd.3 cells express the DREADDs, the previously outlined transfection protocol was implemented. Previous experiments indicated the transfection reagent itself affected resistance, so the control group used in the future analysis included transfected (but not CNO-activated) cells. Dimethyl sulfoxide (DMSO) was used as a solvent for the drug CNO. DMSO with cells was included as another treatment group. A Mann-Whitney U test yielded results indicating that there was a statistically significant difference between the DMSO-only cells and control cells

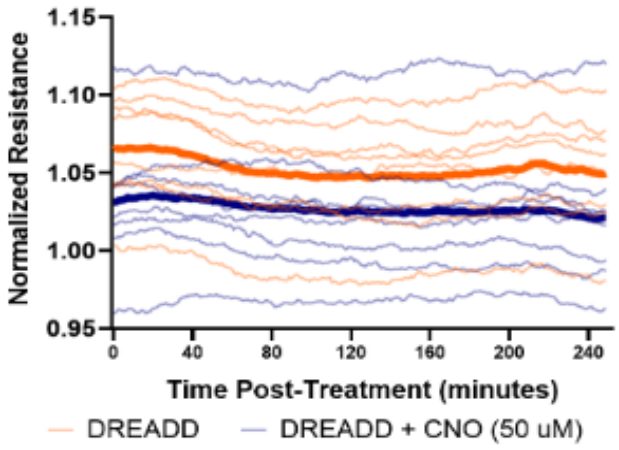

Figure 6. Normalized resistance of treatment groups (untreated DREADD-transfected cells and CNO (50 μM) activated DREADD-transfected cells) post-treatment. Repeated measures two-way ANOVA test (95% confidence interval) yielded a p value of < 0.0001.

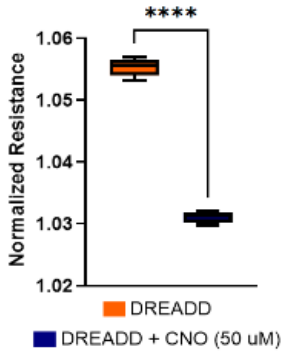

Figure 7. Normalized, averaged resistance of treatment groups (untreated DREADD-transfected cells and CNO (50 μM) activated DREADD-transfected cells) from 50-60 minutes post-treatment. Mann-Whitney test (95% confidence interval).**** *p<0.0001*

(p-value < 0.0001). The DMSO-only cells and unactivated DREADD transfected cells (p-value < 0.0001) also yielded a low p-value indicative of statistical significance. Transfection with DREADDs and activation by CNO was hypothesized to decrease resistance values comparable to the adenylate cyclase inhibitor. As displayed in Figure 6, the control DREADD group retained a higher resistance than the group that was transfected with DREADDs and activated by CNO. To determine the statistical significance of the two groups, a repeated measures two-way ANOVA test yielded a significant p-value of <0.0001. In Figure 7, resistance values between 50 and 60 minutes post-treatment were averaged for each group and compared by a non-parametric Mann-Whitney U test, producing a significant p-value of <0.0001. The statistically significant decrease in resistance further supports our hypothesis that DREADDs can modulate resistance.

Discussion

Computational Netflux model predicts decreased tight junction protein activity after DREADD treatment

The computational model provided strong theoretical support for our hypothesis. Although the model indicated that activating DREADD-transfected BBB endothelial cells would lower claudin-5 expression, it did not provide the exact mechanism of action. The assumption was made that it acted through lowered adenylate cyclase levels. Through these assumptions, the model was modified to reflect this signaling pathway and indicated that tight junction resistance decreases after treatment and activation by DREADDs.

Immunofluorescence Staining verifies decreased expression of tight junction protein claudin-5 after DREADD treatment

Claudin-5 is a crucial tight junction protein for brain endothelial cells and aids in maintaining BBB selectivity. Therefore, reducing claudin-5 expression/activity, to allow for a more permeable barrier, was a key goal. Through IF staining, lower amounts of claudin-5 were observed in DREADD-transfected, CNO-activated cells compared to untreated cells. Similarly, lower amounts of claudin-5 were observed in cells treated with the adenylate cyclase inhibitor drug. The drug inhibits adenylate cyclase, which is the same mechanism DREADDs perform to decrease cAMP. Therefore, these similar results validate the efficacy of the DREADD treatment.

Electric Cell-Substrate Impedance signifies increased bEnd.3 monolayer permeability after DREADD treatment

When conducting our ECIS experiments, a large number of replicates were included to account for variability in the resistance of individual wells. Additionally, 5 different CNO concentrations, ranging from 5 uM to 100 uM, were applied. However, the resistance values did not linearly decrease as CNO concentration increased as expected. Rather, the greatest decrease in resistance occurred with the 50 uM CNO concentration. These results, in combination with those from previous experiments, led to the belief that CNO concentrations might produce a biphasic effect. Another interesting finding of note is the time scale of DREADD activation through CNO. Literature indicates that CNO-activated DREADDs act on a timescale between 1 and 6 hours.[22] However, through experiments, the effects of CNO occurred within the first hour of drug application. One potential explanation for this discrepancy is that most literature on CNO to activate DREADDs is based on *in vivo* work, while all our work was done *in vitro*. If *in vivo* experiments behave in a similar nature to the *in vitro experiments,* it is likely that our treatment only impacts BBB permeability for a short period as resistance measurements return to baseline within three hours of drug application. This is clinically advantageous as persistent permeability decreases in the BBB have negative impacts.

Design Assumptions and Limitations

Regarding the computational model, it was assumed that the claudin-5 activity was lowered due to DREADD-induced lowered adenylate cyclase levels, but the exact mechanism remains unknown. In the experimental design, it was assumed that the behavior of the cell monolayer *in vitro* would be representative of the BBB *in vivo*. However, the behavior of the BBB can differ from that of the cell monolayer, as it is a much simpler version. In real human and animal brains, the BBB is formed from brain pericyte and astrocyte cells, in addition to endothelial cells.[23] We were limited in assessing the validity of this assumption because we were unable to perform *in vivo* experiments. Relatedly, a large constraint on our work was time. After months of testing and refining experimental procedures, each IF staining and ECIS experiment was ~ a 5-day process. Therefore, there was a limited number of experiments that could be performed. Additionally, for over a month, the Price lab faced contamination issues that also impacted the experimental timeline. Time constraints prevented the optimization of some procedures, such as transfections.

Future Implications

There are many opportunities for improvement and expansion relating to the work summarized in this paper. Regarding *in vitro* work, more experiments should ideally be conducted to confirm that CNO-activated DREADDs experience a dose-dependent response (through their resistance values). Our work only tested CNO concentrations on the micromolar scale, but assessing the effects of CNO concentrations on both the nanomolar and millimolar scales would be beneficial to understanding the effects of CNO. Analyzing the effect of CNO-activated DREADDs on other tight junction proteins (zonula occludens, occludin, etc.) could also elucidate more about the mechanism by which the hM4Di DREADD affects the resistance. Additionally, it would be beneficial to perform experiments on a more complex version of the BBB, which could be formed by culturing multiple cell types to more accurately mimic the BBB.[23]

Depending on the results of future *in vitro* work, *in vivo* experiments could be performed to assess the efficacy of DREADDs in actual brains. When applying DREADDs to animal models, it would be important to examine exactly how much resistance decreases and for exactly how long before returning to baseline. Additionally, it would be crucial to determine the toxicity and abilities of CNO at different concentrations.

Regarding broader impacts, this work expands on previous applications of DREADDs as chemogenetic receptors[24,25]. This work repurposes DREADDs which are typically used to study neuronal activity and behavior. By using DREADDs as a therapeutic platform to modulate barrier function, a broader range of research projects could be prompted to investigate alternative novel applications. This work assists in determining the potential applications and limitations of DREADDs. The end-goal drug delivery platform has the potential to deliver drugs to the brain

tissue for many applications, such as delivering medication to slow neurological disease progression. In the future, if our platform is successfully developed, it will provide a method for controlling the size and duration of BBB permeability, advancing the nanopharmaceutical field. Additionally, regarding the impact on patients suffering from neurological disease, this platform has the potential to improve the efficacy of treatments and increase quality of life.

Materials and Methods

Computational Model

To assess the efficacy of the hM4Di DREADD in altering BBB permeability, the effects of DREADDs on cell signaling pathways were simulated. This was done by utilizing a computational model, Netflux, from the Saucerman Lab at the University of Virginia to predict how CNO-activated DREADDs would affect cell signaling pathways in endothelial cells.[18]

Cell Culture Protocol

In vitro experiments were conducted using mouse-derived brain endothelial (bEnd.3) cells. To cultivate bEnd.3 cells for these experiments, Dulbecco's Modified Eagle Medium (DMEM) was used with d-glucose, l-glutamine, sodium pyruvate, and fetal bovine serum. Cells were cultured in a T-175 flask inside a 37°C cell culture incubator until 80% confluency was reached.

Preparation and Mechanism of DREADDs

The hM4Di DREADD was used for all DREADD transfections. The hM4Di DREADD plasmid in bacteria was prepared for all experiments.[26] The bacteria were allowed to culture for 8 hours. After, they were transferred to a larger flask in Luria-Bertani broth. Following an overnight culture, the bacteria were centrifuged and pelleted out. A maxi prep, following the Thermo Fisher Scientific protocol was performed to extract the plasmid DNA from the bacteria.[27] The designer drug of the hM4Di DREADD, CNO, was sourced from SelleckChem in a powdered form.[28] To validate the mechanism by which DREADDs function on a cellular level, the adenylate cyclase inhibitor drug SQ 22536 was sourced from Bio-Techne.[21(p22)]

Transfection Protocol

The ThermoFisher Scientific Lipofectamine 3000 kit was used for all DREADD transfections once the bEnd.3 cells reached 70-90% confluency.[29] Calculations following the transfection kit's protocol were performed. After allowing the DREADDs to transfect for 24 hours, the media was aspirated from each well and replaced with 200 uL of fresh media. The cell monolayers were restabilized in the incubator for 24 hours before drug treatments were applied.

CNO Preparation

CNO. in its powdered form, was measured by an analytical balance. Per the measured mass, powdered CNO was dissolved in DMSO for a resulting stock concentration of 10 mM. Further concentrations were prepared by diluting with cell culture media.

Immunofluorescence Staining

Treatment Groups

2 replicates for each condition were included: 1) control with primary antibody only, 2) control with primary and secondary antibodies, 3) DREADD-transfected, 4) DREADD-transfected with 5 uM CNO, 5) DREADD-transfected with 10 uM CNO, 6) DREADD-transfected with 20 uM CNO, 7) DREADD-transfected with 50 uM CNO, 8) DREADD-transfected with 100 uM CNO, 9) 10 uM adenylate cyclase inhibitor drug, 10) 50 uM adenylate cyclase inhibitor drug, 11) DMSO, and 12) DMSO with 100 uM CNO.

Protocol

IF staining labels specific target proteins with a fluorescent dye, allowing for protein expression quantification. bEnd.3 cells were plated on a 24-well plate at 50000 cells/well and proliferated for 24 hours in augmented DMEM before transfection. The transfection protocol was then performed. 48 hours later, the adenylate cyclase inhibitor drug and two concentrations of CNO were applied for 30 minutes. All media was then aspirated and cells were fixed with paraformaldehyde (PFA). Then, cells were washed with phosphate-buffered saline (PBS), bovine serum albumin (BSA), and Tween 20, followed by blocking with

5% normal goat serum (NGS). The primary rabbit anti-claudin-5 antibody was then added at a concentration of 1:5000.[30] After 24 hours, the secondary antibody anti-rabbit alexa fluor 647 at a concentration of 1:1000 with phalloidin at a concentration of 1:2000 was applied. Lastly, the cells were mounted and imaged for claudin-5.

Electric Cell-Substrate Impedance

Treatment Groups

8 replicates for each condition were included: 1) control, 2) DREADD-transfected, 3) DREADD-transfected with 5 uM CNO, 4) DREADD-transfected with 10 uM CNO, 5) DREADD-transfected with 20 uM CNO, 6) DREADD-transfected with 50 uM CNO, 7) DREADD-transfected with 100 uM CNO, 8) 10 uM adenylate cyclase inhibitor drug, 9) 50 uM adenylate cyclase inhibitor drug, 10) DMSO, and 11) DMSO with 100 uM CNO. All treatment groups that required CNO contained an equal volume of DMSO (to minimize the confounding effect of DMSO on resistance).

Protocol

ECIS applies a small non-invasive alternating current across the electrode pattern in each well. The electric potential is measured ~ every 2 minutes, allowing the electric resistance to be quantified in real time. Resistance is a measure of BBB function because as tight junctions loosen, resistance decreases. Accordingly, ECIS resistance measurements are indicative of permeability changes.[31]

The 96-well PET array was sourced from Applied BioPhysics.[32] The plate was coated with poly-L-lysine for 10 minutes prior to electrode stabilization. After, each well was plated at 36000 cells/300 uL media. The cells stabilized and proliferated for 23 hours before transfection. The transfection protocol was then performed. 48 hours later, the treatments were applied. The ECIS machine collected average resistance measurements at 4000 Hz continuously throughout this process, including for 2-3 days post-treatment application.

Author Contributions and Notes

J.M.H., C.R.S., A.T., T.C., C.G., AND R.P. designed research. J.M.H., C.R.S., and A.T. performed research, analyzed data, and wrote the paper. T.C., R.M., and C.G. trained and assisted in performing research.

The authors declare no conflict of interest.

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References

1. Daneman R, Prat A. The Blood–Brain Barrier. *Cold Spring Harb Perspect Biol*. 2015;7(1):a020412. doi:10.1101/cshperspect.a020412
2. Brain Endothelial Cell-Cell Junctions: How to “Open” the Blood Brain Barrier - PMC. Accessed October 4, 2023. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2687937/
3. Pardridge WM. Drug transport across the blood–brain barrier. *J Cereb Blood Flow Metab*. 2012;32(11):1959-1972. doi:10.1038/jcbfm.2012.126
4. Mayeux R, Stern Y. Epidemiology of Alzheimer Disease. *Cold Spring Harb Perspect Med*. 2012;2(8):a006239. doi:10.1101/cshperspect.a006239
5. Alzheimer’s Disease Fact Sheet. National Institute on Aging. Accessed October 27, 2023. https://www.nia.nih.gov/health/alzheimers-disease-fact-sheet
6. Alzheimer’s Disease Facts and Figures. Alzheimer’s Association. Published 2023. Accessed November 25, 2023. https://www.alz.org/alzheimers-dementia/facts-figures
7. Bloom GS. Amyloid-β and Tau: The Trigger and Bullet in Alzheimer Disease Pathogenesis. *JAMA Neurol*. 2014;71(4):505-508. doi:10.1001/jamaneurol.2013.5847
8. biopharma-reporter.com. Biogen opts into Alzheimer’s drug candidate from pact with Denali Therapeutics. biopharma-reporter.com. Published April 13, 2023. Accessed November 10, 2023. https://www.biopharma-reporter.com/Article/2023/04/1

3/biogen-opts-into-alzheimer-s-drug-candidate-from-pact-with-denali-therapeutics
9. Pardridge WM. Treatment of Alzheimer's Disease and Blood–Brain Barrier Drug Delivery. *Pharmaceuticals*. 2020;13(11):394. doi:10.3390/ph13110394
10. Dong X. Current Strategies for Brain Drug Delivery. *Theranostics*. 2018;8(6):1481-1493. doi:10.7150/thno.21254
11. Non-invasive approaches for drug delivery to the brain based on the receptor mediated transport - ScienceDirect. Accessed May 5, 2024. https://www.sciencedirect.com/science/article/pii/S092849311730574X?via%3Dihub
12. Gandhi K, Barzegar-Fallah A, Banstola A, Rizwan SB, Reynolds JNJ. Ultrasound-Mediated Blood–Brain Barrier Disruption for Drug Delivery: A Systematic Review of Protocols, Efficacy, and Safety Outcomes from Preclinical and Clinical Studies. *Pharmaceutics*. 2022;14(4):833. doi:10.3390/pharmaceutics14040833
13. Rapoport SI. Osmotic Opening of the Blood–Brain Barrier: Principles, Mechanism, and Therapeutic Applications. *Cell Mol Neurobiol*. 2000;20(2):217-230. doi:10.1023/A:1007049806660
14. Hersh DS, Wadajkar AS, Roberts N, et al. Evolving Drug Delivery Strategies to Overcome the Blood Brain Barrier. *Curr Pharm Des*. 2016;22(9):1177-1193.
15. Rohani M, Fasano A. Focused Ultrasound for Essential Tremor: Review of the Evidence and Discussion of Current Hurdles. 2017;7(0):462. doi:10.5334/tohm.378
16. Song KH, Harvey BK, Borden MA. State-of-the-art of microbubble-assisted blood-brain barrier disruption. *Theranostics*. 2018;8(16):4393-4408. doi:10.7150/thno.26869
17. kelly. Focused Ultrasound 101: What to expect before, during and after your -. Regional One Health. Published December 22, 2020. Accessed October 19, 2023. https://www.regionalonehealth.org/blog/2020/12/22/focused-ultrasound-101-what-to-expect-before-during-and-after-your-treatment/
18. GitHub - saucermanlab/Netflux: Netflux is a user-friendly software for developing dynamic computational models of biological networks. Models are created in Excel format and then simulated using the Netflux graphical interface. No computer programming is required. Netflux is written in MATLAB, with binary versions available for Windows and MacOS. Accessed April 29, 2024. https://github.com/saucermanlab/Netflux
19. Ishizaki T, Chiba H, Kojima T, et al. Cyclic AMP induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood–brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. *Exp Cell Res*. 2003;290(2):275-288. doi:10.1016/S0014-4827(03)00354-9
20. Zhu H, Roth BL. Silencing synapses with DREADDs. *Neuron*. 2014;82(4):723-725. doi:10.1016/j.neuron.2014.05.002
21. SQ 22536 Supplier | CAS 17318-31-9 | SQ22536. Tocris Bioscience. Accessed May 5, 2024. https://www.tocris.com/products/sq-22536_1435
22. Whissell PD, Tohyama S, Martin LJ. The Use of DREADDs to Deconstruct Behavior. *Front Genet*. 2016;7:70. doi:10.3389/fgene.2016.00070
23. Comparison of Blood-Brain Barrier Models for in vitro Biological Analysis: One Cell Type vs Three Cell Types - PMC. Accessed May 6, 2024. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6980243/
24. Roth BL. DREADDs for Neuroscientists. *Neuron*. 2016;89(4):683-694. doi:10.1016/j.neuron.2016.01.040
25. Use inert small molecules to control neuronal activity (DREADDs) · Benchling. Accessed October 17, 2023. https://benchling.com/pub/dreadds-hm4di
26. Armbruster BN, Li X, Pausch MH, Herlitze S, Roth BL. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A*. 2007;104(12):5163-5168. doi:10.1073/pnas.0700293104
27. Plasmid DNA Maxiprep Kits | Thermo Fisher Scientific - US. Accessed May 6, 2024. https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/plasmid-isolation/plasmid-dna-maxiprep.html

28. CNO (Clozapine N-oxide) | 99.9%(HPLC) | Selleck | AChR agonist. selleckchem.com. Accessed May 5, 2024. https://www.selleckchem.com/products/clozapine-n-oxide.html
29. Lipofectamine™ 3000 Transfection Reagent. Accessed May 5, 2024. https://www.thermofisher.com/order/catalog/product/L3000015
30. Recombinant Anti-Claudin 5 antibody [EPR7583] (ab131259) | Abcam. Accessed May 5, 2024. https://www.abcam.com/products/primary-antibodies/claudin-5-antibody-epr7583-ab131259.html
31. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER measurement techniques for in vitro barrier model systems. *J Lab Autom*. 2015;20(2):107-126. doi:10.1177/2211068214561025
32. 96W10idf PET (96 Well Array) – Applied BioPhysics Inc. Accessed May 5, 2024. https://applied-biophysics-inc.myshopify.com/products/96w10idf-pet