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Chimeric Claudins Provide a Novel Method to Research Neural Tube Defects

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Tight junctions (TJ) play a major role in the formation of various embryonic structures, including the neural tube. Disruptions of claudins (CLDN), a family of proteins critical to the TJ function, has been shown to induce neural tube defects (NTD) in chicken embryos. Clostridium perfringens enterotoxin (CPE) induces NTDs through this mechanism as it inhibits CLDNs 3, 4, 6, 7, 8, 9, 14, and 19, creating a pore in the TJ and causing cell death. CPE broadly targets these CLDNs in a nonspecific manner. Our research utilizes chimeric-claudin (chCLDN) proteins to target individual CLDN interactions, increasing understanding of their specific contributions to NTDs. Using chicken embryos, we have evaluated the role of chCLDN3 when compared to CPE and solvent. *Gallus gallus* chCLDN-3 (GG3) induced NTDs in chicken embryos at a significantly higher rate than negative controls and statistically similar rate to CPE, our positive control. Additionally, GG3 exhibited different NTD patterns from CPE, allowing us to investigate the unique contributions of CLDN3 in NTD formation and establish the value of this research method.

Terms to Know

Chimeric Claudin (chCLDN): A synthetic protein created to interrupt the binding interactions of a specific CLDN type.

Claudin (CLDN): A family of proteins found within the Tight Junction.

Clostridium Perfringens Enterotoxin (CPE): A naturally occurring toxin found in the bacteria *Clostridium Perfringens*. Treatment with CPE has been shown to increase neural tube defect occurrence.

***Gallus Gallus* 3 (GG3):** Also referred to as chCLDN 3, a chimeric claudin created to specifically interrupt the binding interactions of CLDN 3 within chicken embryos.

Tight Junction: A structure that adheres adjacent cells to one another and regulates ion movement.

836: A synthetic protein used to substitute portions of the native CLDN sequence when creating chCLDNs, allowing the chCLDN to maintain proper structure while inhibiting its ability to integrate into the cell membrane.

Introduction

Within the first few weeks of embryonic development, a cylindrical structure called the neural tube (NT) forms. This tube will eventually fold to become the brain and spine. When the NT fails to fold properly, a birth defect called a neural tube defect (NTD) can result. These birth defects occur most

commonly in the cranial (head) and caudal (tail) region of the embryo but can occur anywhere along the body (Cavalli, 2008). Experts estimate that roughly 300,000 babies are born each year with NTDs and are generally classified as anencephaly, spina bifida, or craniorachischisis (Zaganjor et al., 2016). This can be particularly devastating in developing countries, as NTDs account for nearly 1/3 of newborn deaths due to observable birth defects in these areas (Zaganjor et al., 2016).

Due to the prevalence of these birth defects, countless studies have been conducted to explore the many causes of NTDs. Numerous chemicals and substances that can cause NTDs have been identified, many of which have been linked to damage to a family of proteins called claudins (CLDNs) (Gamero-Estevez et al., 2018). The human genome contains 23 different types of CLDNs which play an important role in creating a structure called the tight junction (TJ). This structure enables neighboring cells to adhere to one another (Gamero-Estevez et al., 2018; Taylor et al., 2021) and is essential for numerous important steps in development including proper NT folding (Baumholtz, Gupta, et al., 2017; El Andalousi et al., 2020).

Despite the importance of these proteins, many current research methods are somewhat limited due to non-specific CLDN targeting and the complicated nature of genetic investigations. Using a new tool developed in our lab called chimeric claudins (chCLDNs), we propose a new method to gain additional insights into the CLDN family of proteins and their role in NTDs.

To fully understand the use of these chCLDNs, it is important to understand the general structure of the TJ and past research methods used to study the CLDN family. The TJ is located between cells in certain tissues and forms a barrier that keeps unwanted particles from passing between neighboring cells (Baumholtz et al., 2020). CLDNs are important for this function as they bind into the cell membrane of one cell and create loops that bind to CLDNs in the adjacent cell, creating a gate-like structure across the cells (Sawada, 2013) and controlling ion flow between adjacent cells (Fromm, 2009). This can be seen in Figure 1A.

Clostridium Perfringens Enterotoxin (CPE), a toxin found in certain bacterias, has been used extensively within research as a CLDN inhibitor. This toxin binds somewhat randomly to eight different types of CLDNs, referred to as CPE-sensitive CLDNs (CLDNs 3, -4, -6, -7, -8, -9, -14, and -19), disabling native CLDN-CLDN interactions between cells and leading to ion imbalance and subsequent cell death (Wieckowski et al., 1994).

Due to this induced cell death caused by CLDN disruption, researchers have shown that chicken embryos treated with CPE have higher occurrence of NTDs than normal chicken embryos (Baumholtz et al., 2020) as the cells making up the NT are disrupted. These studies highlight the importance of CLDNs within proper NT development and their role in preventing NTDs. However, because CPE-based methods are unable to specifically target one CLDN type at a time, our ability to understand which types of CLDNs most directly affect NTD formation is limited (Baumholtz et al., 2020). In addition,

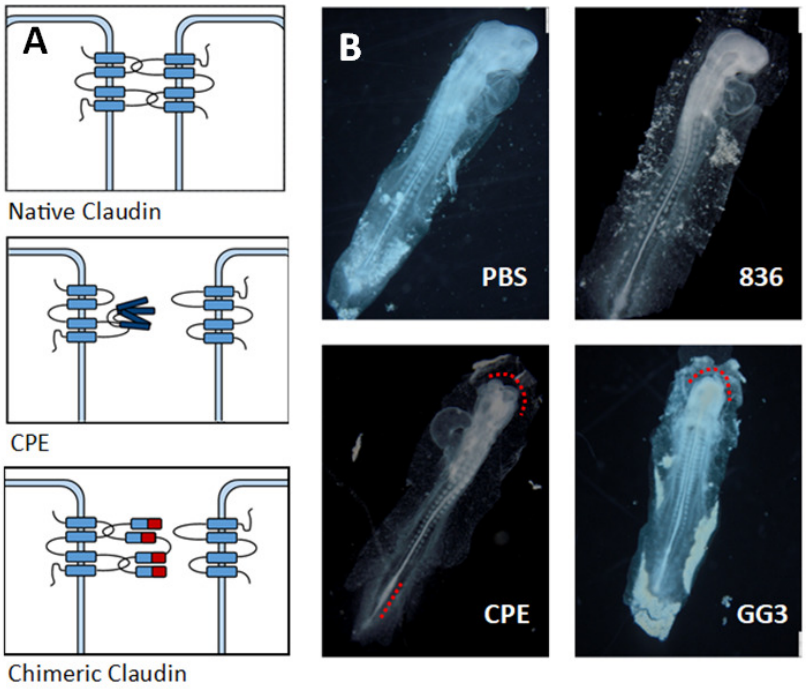

Figure 1. A) Claudins create barriers between neighboring cells that are important for NT development. CPE disrupts CPE-sensitive claudins, leading to an increase in NTDs. chCLDNs allow targeting of a specific CLDN's binding interactions. These are made up of the native CLDN-CLDN binding domain of the CLDN of interest (shown in blue) and a synthetic protein, 836 (shown in red). B) Incubation of developing chicken embryos in agar containing GG3 and CPE induced both cranial (head) and caudal (tail) NTDs. PBS and 836 were used as negative controls

CPE-based methods are restricted to the eight CPE-sensitive CLDNs mentioned above, leaving the contribution of other CLDN families largely uninvestigated. In order to overcome this limitation, genetic knockouts of single CLDN types have been conducted. However, compensation between CLDN types occurred, making it difficult to use genetic methods to identify the contributions of a single CLDN within NT development (Baumholtz et al., 2020).

To overcome these limitations and better understand the individual contributions of various CLDN proteins, we utilized a chCLDN, a lab-synthesized protein that blocks specific types of CLDN interactions (Taylor et al., 2021). Using this method, we inhibited CLDN3 within chicken embryos and observed the results. The specific inhibition of CLDN3 (a CPE-sensitive CLDN) using chCLDNs enables us to compare the efficacy of this method to the currently established CPE protocol. Similarities between these results would validate the use of chCLDN as a valuable method for future studies investigating the role of specific types of CLDN proteins.

Methods

Chimeric Claudin Synthesis

The goal of the chCLDN is to bind to native CLDN proteins and disrupt their ability to bind naturally with other CLDNs (see Figure 1a). To create this protein, we utilized the method outlined by Taylor et al., 2021. After collecting the DNA sequences of our CLDN of interest, the sequences corresponding with the transmembrane regions of the protein were substituted for a synthetic

protein (referred to hereafter as 836), allowing it to maintain the general CLDN structure while preventing binding within the cell membrane. By doing so, the chCLDN has the ability to bind to specific types of CLDNs and interrupt their native interactions across the TJ. This allowed us to target a single CLDN type individually (i.e. CLDN3) and investigate its unique role in NTD formation. Plasmids were then used to produce these proteins followed by amylose resin purification.

The chicken embryo model was chosen for this experiment as the NT is easily accessed and visualized throughout its formation (Ross, 2020). As such, we acquired the amino acid sequences specific to the chicken species (*Gallus gallus*, accession number Q98SR2) to create our chCLDN of interest. CLDN3 was selected due to its relatively high concentration within the NT, making it a good candidate for NTD related mechanisms (Collins et al., 2013). Additionally, since CLDN3 is a CPE-sensitive CLDN, chCLDN3-treated embryos could be more accurately compared to CPE-treated embryos to confirm the efficacy of the chCLDN method.

Embryo Collection and Treatment

37 embryos were treated with inhibitory chCLDN3, hereafter referred to as *gallus gallus* 3, or GG3, using the EC Culture protocol published by Stark and Ross in 2019 (Stark & Ross, 2019). Consistent with this protocol, embryos were removed from their shell after 30-35 hours of incubation, carefully collected, and placed on agar plates containing GG3. This time period was selected as it allowed the embryos to be exposed to GG3 during the time frame when the NT is actively forming (Aaku-Saraste et al., 1996).

In addition to GG3, agar plates contained phosphate-buffered saline (PBS) as a solvent, penicillin-streptomycin antibiotics to prevent bacterial contamination, and albumin (egg white) to provide nutrients for embryonic development. Additional plates were prepared substituting GG3 for similar doses of CPE (27 embryos, positive control), PBS (29, negative control), and 836 (44, negative control). During collection, excised embryos were placed on an agar plate containing one treatment and allowed to incubate in this medium for 24 hours.

Scoring and Analysis

Following this incubation period, each embryo was preserved in formaldehyde, and imaged at 12.5x and 25x magnification (see Figure 1B). The images were then visually examined for NTDs in both cranial and caudal regions and proportions were calculated within each treatment group. Examples of both types of NTDs are presented in Figure 1B. Fisher Exact tests were then conducted to determine the significance of variations observed between the treatments.

Results

Overall, 35.14% of the embryos incubated with GG3 expressed a caudal or cranial NTD (see Figure 2a). This was significantly higher than the proportion of NTDs found in either negative control, with only 6.90% in PBS ($p < 0.01$)

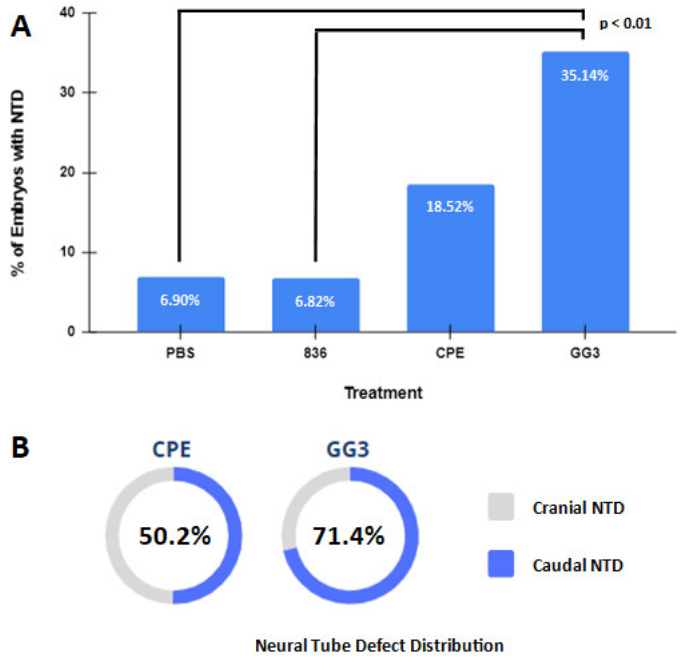

Figure 2. A) Proportions of embryos in each treatment that exhibited a NTD. GG3 significantly increased the rate of NTDs compared to controls, as shown by p-value. B) The location of NTDs varied between CPE and GG3 treatment, with GG3 treated embryos developing caudal NTDs more frequently than cranial NTDs.

and 6.82% in 836 ($p < 0.01$). No significant difference was observed when compared to the positive control, as CPE exhibited NTDs in 18.52% ($p = 0.1699$) of embryos.

CPE-treated embryos exhibited cranial and caudal NTDs at a nearly 1:1 ratio (50.2%), while GG3-treated embryos caused a higher percentage of caudal NTDs over cranial NTDs, with 71.43% of the total NTDs being caudal (see Figure 2B). Sample size restrictions prevented proper calculation of significance between these groups, warranting further research to investigate these patterns.

Discussion and Conclusion

These findings provide evidence that chCLDNs offer an effective method to study the role of CLDNs within NT development. Compared to negative controls, GG3 significantly increased the rate of NTD formation within developing chicken embryos. This confirms existing evidence that CLDNs play a significant role in embryonic NT formation (Baumholtz, Simard, et al., 2017) and establishes the validity of this method. In this regard, the control involving 836 confirms that the increase of NTDs is due to the interruption of CLDN binding, rather than interference from the synthetic building blocks used to create the chCLDNs.

In addition, the lack of significant difference between NTD incidence in embryos treated with GG3 vs CPE strengthens the argument for chCLDNs' use in NTD research, providing evidence that they may be used in future studies to inhibit CLDN-specific interactions without sacrificing one's ability to induce NTDs effectively.

Although the difference of overall rate of NTDs was not statistically significant between CPE and GG3, there was a variation in the ratio of cranial vs caudal NTDs between treatments. This data suggests that specifically disrupting CLDN3 may show patterns different from those observed when disrupting all CPE-sensitive CLDNs collectively. Using this method to study additional CLDN types, it is likely that other patterns will arise, increasing our ability to understand the role of these proteins within NT formation.

By more fully understanding mechanisms of NT formation, we will be able to investigate ways to explore new mechanisms of NTD treatment and prevention.

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References

Aaku-Saraste, E., Hellwig, A., & Huttner, W. B. (1996). Loss of Occludin and Functional Tight Junctions, but Not ZO-1, during Neural Tube Closure—Remodeling of the Neuroepithelium Prior to Neurogenesis. *Developmental Biology*, *180*(2), 664–679. https://doi.org/10.1006/dbio.1996.0336

Baumholtz, A. I., De Marco, P., Capra, V., & Ryan, A. K. (2020). Functional Validation of CLDN Variants Identified in a Neural Tube Defect Cohort Demonstrates Their Contribution to Neural Tube Defects. *Frontiers in Neuroscience*, *14*(664). https://doi.org/10.3389/fnins.2020.00664

Baumholtz, A. I., Gupta, I. R., & Ryan, A. K. (2017). Claudins in morphogenesis: Forming an epithelial tube. *Tissue Barriers*, *5*(4), e1361899. https://doi.org/10.1080/21688370.2017.1361899

Baumholtz, A. I., Simard, A., Nikolopoulou, E., Oosenbrug, M., Collins, M. M., Piontek, A., Krause, G., Piontek, J., Greene, N. D. E., & Ryan, A. K. (2017). Claudins are essential for cell shape changes and convergent extension movements during neural tube closure. *Developmental Biology*, *428*(1), 25–38. https://doi.org/10.1016/j.ydbio.2017.05.013

Cavalli, P. (2008). Prevention of Neural Tube Defects and proper folate periconceptional supplementation. *J Prenat Med*, *2*(4), 40–41.

Collins, M. M., Baumholtz, A. I., & Ryan, A. K. (2013). Claudin family members exhibit unique temporal and spatial expression boundaries in the chick embryo. *Tissue Barriers*, *1*(3), e24517. https://doi.org/10.4161/tisb.24517

El Andalousi, J., Khairallah, H., Zhuang, Y., Ryan, A. K., & Gupta, I. R. (2020). Role of Claudins in Renal Branching Morphogenesis. *Physiological Reports*, *8*(18). https://doi.org/10.14814/phy2.14492

Fromm, M. (2009). Molecular Structure and Function of the Tight Junction: From Basic Mechanisms to Clinical Manifestations. *Annals of the New York Academy of Sciences*, *1165*(1), 1–6. https://doi.org/10.1111/j.1749-6632.2009.04925.x

Gamero-Estevez, E., Baumholtz, A. I., & Ryan, A. K. (2018). Developing a link between toxicants, claudins and neural tube defects. *Reproductive Toxicology*, *81*, 155–167. https://doi.org/10.1016/j.reprotox.2018.08.008

Ross, M. M. (2020). A Comprehensive Comparison of Teratogenic Compounds Known to Induce Neural Tube Defects in the Chicken Embryo. *Theses and Dissertations*, *9257*. https://scholarsarchive.byu.edu/etd/9257

Sawada, N. (2013). Tight junction-related human diseases. *Pathology International*, *63*(1), 1–12. https://doi.org/10.1111/pin.12021

Stark, M. R., & Ross, M. M. (2019). The Chicken Embryo as a Model in Developmental Toxicology. *Methods in Molecular Biology*, *1965*, 155–171. https://doi.org/10.1007/978-1-4939-9182-2_11

Taylor, A., Warner, M., Mendoza, C., Memmott, C., LeCheminant, T., Bailey, S., Christensen, C., Keller, J., Suli, A., & Mizrachi, D. (2021). Chimeric Claudins: A New Tool to Study Tight Junction Structure and Function. *International Journal of Molecular Sciences*, *22*(9), 4947. https://doi.org/10.3390/ijms22094947

Wieckowski, E. U., Wnek, A. P., & McClane, B. A. (1994). Evidence that an approximately 50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound Clostridium perfringens enterotoxin. *Journal of Biological Chemistry*, *269*(14), 10838–10848. https://doi.org/10.1016/s0021-9258(17)34135-2

Zaganjor, I., Sekkarie, A., Tsang, B. L., Williams, J., Razzaghi, H., Mulinare, J., Sniezek, J. E., Cannon, M. J., & Rosenthal, J. (2016). Describing the Prevalence of Neural Tube Defects Worldwide: A Systematic Literature Review. *PLoS One*, *11*(4), e0151586. https://doi.org/10.1371/journal.pone.0151586