Molecular mechanism of OXT/OXTR pathway manipulation in mouse and iPSC hypothalamic neuron models

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Abstract

Oxytocin (OXT) is a neuropeptide linked to empathy in mammals. Dysregulation of OXT and oxytocin receptor (OXTR) leads to a disruption in empathic behavior, namely decreasing the level of empathy observed. While the OXT pathway is not understood, and further research must be done to elucidate the underlying mechanisms leading to empathic behavior, Lopatina et al. (2012) suggest an integral link between OXT and the protein PKC-CD38/ADP-ribosyl cyclase cascade in OXT neurons. Using this knowledge, the goal of this proposal is to explore novel methods for examining the influences of the suggested pathway through manipulation of its components and its resulting changes in empathic behavior. We hypothesize that these approaches will reveal new methods for the modification of empathic behavior as well as additional treatment potentials for diseases that affect empathic behavior, including autism and William's syndrome.

Background

Empathy in a broad sense refers to the reactions of one individual to the observed experiences of another. These reactions tend to have two main components: a cognitive reaction, the ability to understand what another person is experiencing, and an emotional reaction, actually feeling what the other is feeling (1). These reactions can embody many faculties of emotion, including self-awareness, morality, compassion, and even prosocial behavior.

While we are able to observe and measure empathic behavior, the biological mechanism for its origin is still unknown. One possible pathway leading to empathic behavior involves the neuropeptide oxytocin (OXT). Oxytocin itself is produced in nerve cell bodies in the hypothalamus and is secreted from axon endings into both the brain and bloodstream, acting as a neurotransmitter and a hormone. It is known to play a role in reproductive functions, as it is secreted to the uterus and mammary glands during childbirth and lactation (2). Oxytocin also has social implications, playing a role in pair-bond formation, attachment, and prosocial responding (3,4). Specifically, an epigenetic modification of the oxytocin gene has been shown to have implications in human sociability, with human subjects displaying deficits in facial and emotional recognition and lowered brain activity in regions related to sociability (5). Oxytocin's involvement in human sociability therefore provides support for oxytocin pathway molecules also having an influence on social behavioral phenotypes, specifically empathy.

Looking deeper into the relationship oxytocin plays in empathy, there are a few key downstream regulators that have been found to be involved in the release of oxytocin and subsequent empathic behavior. Two of these molecules are protein kinase C (PKC) and glycoprotein CD38, which positively regulate oxytocin release (6,7,8). PKC is a protein kinase enzyme that is involved in controlling the function of other intracellular proteins. Evidence suggests PKC therefore functions in the oxytocin pathway by signaling the activation of downstream CD38 (9,10). Furthermore, CD38 itself is a multifunctional enzyme that catalyzes the hydrolysis of cyclic ADP-ribose (cADPR) from NAD+ and ADP-ribose. This is essential for signaling intracellular calcium release, ultimately leading to the desired oxytocin release (11). At the behavioral level, CD38 knockout mice have been shown to display deficits in learning and memory, which are similar to behaviors displayed in autism (12). Additionally, attention and social eye cue deficits have been found in human infants homozygous for a CD38 allele associated with a reduction in oxytocin release, similarly suggesting a possible link to autism and a lack of empathy (13). These studies give insight into CD38's crucial role in empathic behaviors. A third protein in the oxytocin pathway following PKC and CD38 is ryanodine receptor 3 (RyR3). RyR3 is an intracellular calcium channel and receptor that has been proposed to activate in response to CD38 signaling, which ultimately releases calcium and signals subsequent oxytocin release (14,15). The way PKC, CD38, and RyR3 aid in the activation and inhibition of the oxytocin pathway and empathic behavior has yet to be understood. The focus of this study is to explore these factors more closely, looking at each of these downstream proteins and the oxytocin receptor itself, which has been shown to induce a production of more oxytocin when activated (16). By understanding how all of these pieces fit together, we will be able to better understand how empathy is formed at a molecular level and how this translates to the behavioral level, providing valuable insights for disorders which lack empathy, such as autism, or disorders with an abundance of empathy, such as William's syndrome.

Relevance

Broader Relevance: The oxytocin pathway is a proposed mechanism of action that underlies prosocial behavior, including empathy. Previous studies have pointed to a few key mediator proteins that may play a role in the pathway, which ultimately leads to the elicitation of empathy. However, there has been no conclusive research to fully support this idea. The findings within this proposal will aid in expanding our knowledge on the specific molecular messengers that work in the oxytocin pathway, as well as how they regulate and contribute to empathic behaviors. Autism Spectrum Disorder (ASD) is characterized as a range of neurodevelopmental disorders, affecting every 1 in 68 children in the United States alone (17). Many children who suffer from ASD show deficits in empathic behavior, such as issues with attention, social gaze, and emotional perspective taking in empathy tasks (18). Currently, there is no known cure or sufficient treatment for autism. This study will contribute to understanding how regulating the oxytocin pathway and its messengers may be a feasible therapeutic strategy in aiding treatment for ASD patients as well as other empathy-related disorders.

Intellectual Merit: In this proposal, we will explore the role oxytocin and its downstream signaling molecules play in altering empathy, namely prosocial behavior. By exploring a mechanism involved in the creation of empathetic behavior, we may determine what leads to the pathogenesis of empathy-related disorders, such as autism or William's syndrome. These discoveries will contribute to the field of neuroscience as a whole by helping us understand at a cellular level what goes awry in such disorders and how this leads to subsequent changes in behavior, ultimately suggesting possible therapy targets that can be implemented for empathy-related disorders.

Specific Aims

The overall goal of this project is to further explore the role oxytocin plays in empathy. Specifically, this study will test the role oxytocin and its pathway plays in regulating empathic behavior. It is hypothesized that oxytocin regulates empathic behavior in hypothalamic neurons.

1. To vary oxytocin and its receptor to see how it modifies empathic behavior:

Two approaches, genetic and pharmacological, will be used to over- and under-express oxytocin, its receptor, and a genetic variant of the receptor, rs237887, which has been shown to be associated with an increase in empathy (19). First, overexpression models will be created using transgenic mice and agonist addition mice. Second, underexpression models will be created using knockout and antagonist addition mice. Once these models are created, they will be evaluated in how they change the activity of three oxytocin downstream messengers: PKC, CD38, and RyR3 and how this then results in differences in empathic behavior as a whole.

2. To manipulate the oxytocin pathway messengers to observe how it affects empathic behavior:

Two approaches will be used to analyze the over- and underexpression of oxytocin pathway messengers PKC, CD38, and RyR3. The overexpression models will utilize transgenic mice and pathway messenger agonists, and the underexpression models will utilize knockout and shRNA mice, as well as pathway messenger antagonists/inhibitors. These models will be studied using appropriate techniques to evaluate behavioral and physiological differences between overexpression, underexpression, and normal levels of the proposed pathway messengers, ultimately determining how these differences affect empathic behavior.

3. To obtain iPSC hypothalamic patient neurons to observe, modify, and restore OXTR/OXT pathway functionality:

The previous two aims shall be combined with the overall goal of examining the mechanistic processes involved in prosocial behavior in patient models. Hypothalamic neurons will be derived from iPSCs via adult human fibroblast reprogramming and differentiation. Fibroblasts shall be obtained from four groups; Autism patients (low OXT conc.), William's Syndrome patients (high OXT conc.), "healthy" individuals with severe clinical depression (low OXT conc.), and individuals with normal OXT levels. These four models will present a strong variance for OXT concentrations, providing an effective set of models for examining OXT/OXTR pathway expression in patient models. Once OXT expression is determined in each model, similar manipulations performed in the previous two aims of the study will be completed to determine OXT expression accuracy relative to previous results. In addition, attempts will be made to restore OXT/OXTR and pathway expression to normally expected levels in the diseased models.

Research Design and Methods

Aim 1: To vary oxytocin and its receptor to see how it modifies empathic behavior.

Rationale: It is necessary to determine how over- or underexpression of oxytocin and its receptor differ in order to determine which form drives empathic behavior, and furthermore how this relates to empathy-related disorders such as autism or William's syndrome. Secondly, by modifying the levels of oxytocin and its receptor, we will be able to explore how this affects downstream molecules and how these molecules also play a role in the creation of empathic behavior. Mice will be studied for both of these approaches as they have genetic, biological, and behavioral characteristics closely resembling that of humans. Specifically, they are able to demonstrate measurable empathic behavior (20).

Design and Method

1. Developing and verifying overexpression models:

Two approaches, genetic and pharmacological, will be used to create an overexpression of oxytocin, its receptor, and receptor variant rs53576, which is associated with an increase in empathy. Mice with unaltered oxytocin levels will serve as the control. The genetic model will be created using transgenic mice with the transcriptional control of three different promoters: synapsin 1 promoter, oxytocin promoter, and oxytocin protomer expressing Cre recombinase. Different promoters will be used in order to vary the location and time of expression. The human synapsin 1 gene promoter, which is expressed in neurons, will be used as a general promoter to show overall oxytocin localization throughout the entire brain (21). The oxytocin promoter will be used as a hypothalamus specific promoter to show oxytocin localization specifically in the hypothalamus (22). Lastly, the oxytocin promoter expressing Cre recombinase will be used as a hypothalamus time specific promoter to show time sensitive oxytocin changes before and after transgene addition (23). The second approach in creating an overexpression model will be made by intravenously injecting OXTR agonist Carbetocin into mice, serving as an alternate way to overexpress oxytocin receptor pharmacologically (24). To confirm mice have been successfully created, Northern and Western blot analyses will examine transgene expression and protein levels, respectively.

Prediction: We expect to see normal RNA in the control mice and mutant RNA in the overexpression models. We also expect to see normal protein in the control models and overexpressed protein in the overexpression models. If these results are not obtained, the models have not been successfully created.

2. Developing and verifying underexpression models:

Two approaches, genetic and pharmacological, will be used to underexpress oxytocin and its receptor. Mice with unaltered oxytocin levels will serve as the control. The genetic model, knockout mice, will be created using Cre recombinase to create conditional knockout mice by excising the DNA between the two loxP sites surrounding OXT and OXTR (25). Secondly, the OXTR antagonist Atosiban will be intravenously injected into separate mice to serve as an alternate way of creating a pharmacologically induced underexpression model (26). To confirm these mice have been successfully created, Northern and Western blot analyses will examine transgene expression and protein levels, respectively.

Prediction: We expect to see normal RNA in the control mice and mutant RNA in the underexpression models. We also expect to see normal protein in the control models and underexpressed protein in the underexpression models. If these results are not obtained, the models have not been successfully created.

3. Studying activity changes in downstream molecules of the oxytocin pathway:

Mice will be tested for PKC, CD38, and RyR3 activity, each a downstream messenger in the oxytocin pathway. Given PKC is the first messenger following the oxytocin receptor, it will be studied first. PKC activity will be assessed using the PKC Kinase Activity Kit (ab139437), which uses a PKC specific substrate that becomes phosphorylated by PKC after the addition of ATP. The assay is developed with tetramethylbenzidine (TMB) substrate and color develops proportional to the PKC activity existing in the sample (27). The second messenger in sequence is CD38. CD38 produces diphosphoribose (ADPR), the activity of which will be assessed using reverse-phase HPLC. Reverse-phase HPLC functions to separate CD38 from ADPR, with the absorbance of ADPR measured and quantified to show CD38 activity (28). The last messenger in sequence is RyR3. RyR3 channel activity will be measured using a custom designed amplifier by Fill et al. (29) and a TL-1 DA/AD interface via patch-clamp recording (30).

Prediction: Overexpression mice are expected to all show an increase in the activity of PKC, CD38, and RyR3 compared to that of the control. Conversely, underexpression models are expected to show the opposite, reduced activity of PKC, CD38, and RyR3.

4. Studying changes in empathy behavior:

Mice will be tested for empathic behavior differences using three tests. The first test will measure allogrooming, or the amount of consolation grooming exhibited towards a cagemate after the cagemate experiences a stress in the form of a shock or tone (31). The second test will measure social activity preferences, which are measured by whether the mouse chooses to remain in an empty cage or a cage with other mice, therefore choosing to be social. Lastly, goal-directed behavior will be measured by observing the behavior of a mouse to free a conspecific from a trapped restrainer in a goal-directed fashion (20).

Prediction: Allogrooming, social activity, and goal directed behavior are all expected to increase in overexpression models. Conversely, underexpression models are expected to show decreased levels of allogrooming, social activity, and goal-directed behavior. Results from Aim 1 will provide evidence for the hypothesis that oxytocin plays an important role in generating empathic behaviors as well as affecting downstream molecules in the oxytocin pathway, which will be further explored in Aim two.

Aim 2: To manipulate the oxytocin pathway messengers to observe how it affects empathetic behavior.

Rationale: It is necessary to determine how an over- or underexpression of the oxytocin pathway messengers will differ in order to observe how the pathway is interconnected, how they affect empathetic behavior, and how this relates to empathy-related disorders such as autism. By modifying the levels of the oxytocin pathway molecules, we will be able to explore how this affects each molecule, and how these molecules play a role in empathic behavior. When this is analyzed, it will allow the appropriate form to be studied in order to understand the mechanism of the signaling pathway and how this relates to behavioral changes in empathy.

Design and Method

1. Developing and verifying overexpression models: Similar to Aim 1, two approaches will be used to create an overexpression of oxytocin pathway messengers: PKC, CD38, and RyR3; transgenic mice and agonist addition mice. Mice with unaltered oxytocin levels will serve as the control. Transgenic mice will be created under the transcriptional control of three different promoters in hypothalamic neurons: PKRCA gene promoter, CD38 gene promoter, and GRCm38 (RyR3) gene promoter. The second approach in creating an overexpression model will be made by intravenously injecting agonists I kappa B kinase (PKC), Endothelin-1 (CD38), and Suramin (RyR3). Currently, there has not been any recent research for either overexpression model that has looked into such transgenic studies, or the utilization of such agonists on the pro-

posed oxytocin pathway messengers. Thus, it is important to implement this novel approach for further analyzation. To confirm mice have been successfully created, Northern and Western blot analyses will examine transgene expression and protein levels, respectively.
Prediction: We expect to see normal RNA in the control mice and mutant RNA in the overexpression models. We also expect to see normal protein in the control models and overexpressed protein in the overexpression models. If these results are not obtained, the models have not been successfully created.

2. Developing and verifying underexpression models:

Similar to Aim 1, two approaches will be used to create an underexpression of oxytocin pathway messengers PKC, CD38, and RyR3; knockout and shRNA mice, as well as antagonist addition mice, will be used to create an underexpression of oxytocin and its receptor. Mice with unaltered oxytocin levels will serve as the control. Knockout mice will be created using Cre recombinase to create conditional knockout mice by excising the DNA between the two loxP sites surrounding PKRCA, CD38, and RyR3 genes. Second, shRNA mice will be created using a viral vector containing the shRNA, which will be injected in vivo using an integrase-deficient lentivirus (32). The shRNA will be transcribed in the nucleus by polymerase on the PKRCA, CD38, and GRCm38 (RyR3) promoter genes, which will then silence the target genes. The third approach in creating an overexpression model will be made by intravenously injecting antagonists/inhibitors Calphostin C (PKC), 8-bromo-cADPR (CD38), and Dantrolene (RyR3). Research has shown that Calphostin C and 8-bromo-cADPR led to reduced oxytocin release as well as reduced CD38 sensitivity, indicating the role of PKC and CD38 in the signaling cascade (10, 16). However, research has yet to be done analyzing shRNA mice and RyR3 antagonists for the pathway messengers. To confirm these mice have been successfully created, Northern blot and Western blot analyses will examine gene expression and protein levels, respectively.
Prediction: We expect to see normal RNA in the control mice and mutant RNA in the underexpression models. We also expect to see normal protein in the control models and underexpressed protein in the underexpression models. If these results are not obtained, the models have not been successfully created.

3. Studying activity of downstream molecules:

Similar to Aim 1, mice will be tested for PKC, CD38, and RyR3 activity. PKC activity will be assessed using the PKC Kinase Activity Kit (ab139437), which uses a PKC specific substrate that becomes phosphorylated by PKC after the addition of ATP. The assay is developed with tetramethylbenzidine (TMB) substrate and color develops proportional to the PKC activity existing in the sample. CD38 activity will be assessed using reverse-phase HPLC. RyR3 channel activity will be measured using a custom designed amplifier by Fill et al. (29) and a TL-1 DA/AD interface via patch-clamp recording.

Prediction: Overexpression mice are expected to all show an increase in the activity of PKC, CD38, and RyR3 compared to that of the control. Conversely, underexpression models are expected to show the opposite, reduced activity of PKC, CD38, and RyR3.

4. Studying changes in empathetic behavior:

Similar to Aim 1, mice will be tested for empathic behavior differences using allogrooming, social activity preferences, and goal directed behavior tests.
Prediction: Allogrooming, social activity, and goal directed behavior are all expected to increase in overexpression models. Conversely, underexpression models are expected to show decreased levels of allogrooming, social activity, and goal-directed behavior. Results from Aim 2 will provide evidence for the hypothesis that the proposed oxytocin pathway messenger proteins create a signaling cascade, and play an important role in regulating empathic behavior.

Aim 3: To obtain iPSC hypothalamic patient neurons to observe, modify, and restore OXTR/OXT pathway functionality.

Rationale: It is necessary to determine if similar results can be replicated in iPSC hypothalamic neurons, as replication of these physiological characteristics in iPSC neurons can aid in further modeling techniques of both empathetic behavior and diseases affected by the OXT pathway. iPSC hypothalamic neurons alone are an effective model, as seen in previous studies by Wang et al. (33, 34); however, the transplantation of these iPSC neurons into mouse models will enhance the ability to replicate behavioral effects induced by changes in the OXT pathway.

Design

1. Developing iPSC hypothalamic neurons:

Skin biopsies from healthy, variant rs53576, ASD, and William's syndrome (WS) patients will be obtained and developed into fibroblasts, after which they will be transformed into pluripotent stem cells (35). This process will be performed as outlined in two papers by Wang et al. (33, 34). Comparisons will be made with known patient hypothalamic neurons in each of the four conditions to ensure proper differentiation occurred. Comparisons will be made between the control and iPSC developed hypothalamic neurons using a one-way ANOVA test for statistical significance.

Prediction: Developed iPSC will show proper characteristics of hypothalamic neurons as seen in healthy, variant, WS, and ASD patients. Determining the relative accuracy of these characteristics will be based off of previously established knowledge of typical neurons. If expected characteristics of each neuron are not seen, then the differentiation did not properly occur and the process should be repeated.

2. Comparison of physiological effects in iPSC derived hypothalamic neurons to previous results:

Further examination of the successfully developed and differentiated iPSC hypothalamic neurons will be performed. Using the methods of three previous assays to test for the physiological expression of CD38, RyR3, and PKC, comparisons will be made between iPSC derived hypothalamic neurons and the previously examined results found from aims 1 and 2. Comparisons will be made using a one-way ANOVA test for statistical significance
Prediction: Assays examining PKC, CD38, and RyR3 for the various iPSC hypothalamic neurons should fall in line with expected results for control neurons as seen in patients. If the results do not follow this, testing should be performed again and, possibly, the creation of new iPSC hypothalamic neurons.

3. Transplanting iPSC derived hypothalamic neurons into mouse model and testing for pathology:

Investigation of derived iPSC hypothalamic neurons in a mammalian model is critical both for replication and for effective pathway manipulation strategies. Mouse models will be used for their effectiveness in expressing behavioral and physiological aspects of the various conditions listed. Czupryn et al.'s methodology will be used for transplantation of iPSC neurons into the hypothalamus. iPSC hypothalamic neurons will be inserted into mouse models via micro-transplantation directly into the medial hypothalamus of postnatal mice (36). Following successful transplantation, various methodologies as previously discussed in aims 1 and 2 will be performed to test for the success of replication for the iPSC hypothalamic neuron mouse model and previously examined mouse models. Comparisons will be made between the control mice models and iPSC developed hypothalamic neuron mouse models using a one-way ANOVA test for statistical significance.

Prediction: After transplanting the WT, ASD, OXTR variant, and WS iPSC hypothalamic neurons into the mouse models successfully, we expect to see similar pathological and behavioral expression of each respective disease in the mice. Allogrooming, social activity, and goal-directed behavior will again be observed. These behaviors are all expected to increase in the variant and WS model. Conversely, the autism model is expected to show decreased levels of allogrooming, social activity, and goal-directed behavior. If not, an error was made during injection and the process should be repeated. If proper pathological and behavioral expression occurs, then behavioral and physiological tests may proceed. The results of aim 3 should provide an effective method for modelling empathy in human iPSC hypothalamic neuron models.

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