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Serine Proteases and Their Inhibitors Affect the Rate of GnRH Neuronal Migration in the Embryonic Chick Model: A Thesis Proposal

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ABSTRACT

Development in an organism is a highly complex task that requires a specific sequence for proper function. One of these tasks is neuronal migration and the extracellular cues that help to guide neurons to their appropriate locations. If neurons do not migrate to their appropriate locations, then there are abnormal neural connections, which can result in neuronal migration disorders (NMD). It has been shown that the inhibition and stimulation of migration may be balanced due to effects of serine protease inhibitors and serine proteases. Serine proteases and their inhibitors can alter the various chemoattraction-signaling cues and thereby modulate neuronal migratory processes. Furthermore, there exists a delicate balance between serine proteases and their inhibitors to modulate migration of neurons within development. Thus, by understanding how the balance of serine protease and serine protease inhibitor activity works, one can further understand how these changes affect neuronal migration in development.

Experiments to test in vivo neuronal migration of chick GnRH neurons during embryogenesis were investigated to elucidate the role these proteins play in migration. Protein coated beads were inserted and GnRH levels measured to control. Such experimentation is crucial, as these studies are the first to explore the role of proteases in neural migration in an in vivo model.

These findings indicate that the ability to initiate migration lies inherently within the neuron itself such that changes of the extracellular matrix composition are irrelevant in the initial timing. Neurons entering the CNS earlier in trypsin-treated embryos indicates continuous movement may be a cell autonomous event with trypsin-induced proteolysis. In other words, the ability of GnRH neurons to migrate across glial boundaries and through the brain to its target site is independent of further maturation of the neuron or the brain. Taken together, this study can provide knowledge regarding these proteins and their relation to development. This knowledge can potentially lead to therapies that can provide ameliorative care for patients suffering with NMD.

Keywords

Signaling transduction, Biochemistry, Proteins, Receptors, Neuronal migration, Serine protease, Serine protease inhibitor.

Introduction

Background and Significance

Development in an organism is a period that is made up of highly complex and specific tasks that have to occur in sequence for proper function. One of these tasks is neuronal migration and what extracellular cues are secreted into the extracellular matrix (ECM) that help to guide and coordinate neurons to their appropriate locations. Although this process is poorly understood, specific proteins, called serine proteases and serine protease inhibitors, might help in modulating the migration of these neurons during development by selectively inactivating their target proteases [1].

Figure 1 below shows the reaction mechanism for serine proteases. The first step is a proton transfer that the histidine does from the serine residue, which acts to make the serine more nucleophilic [2].

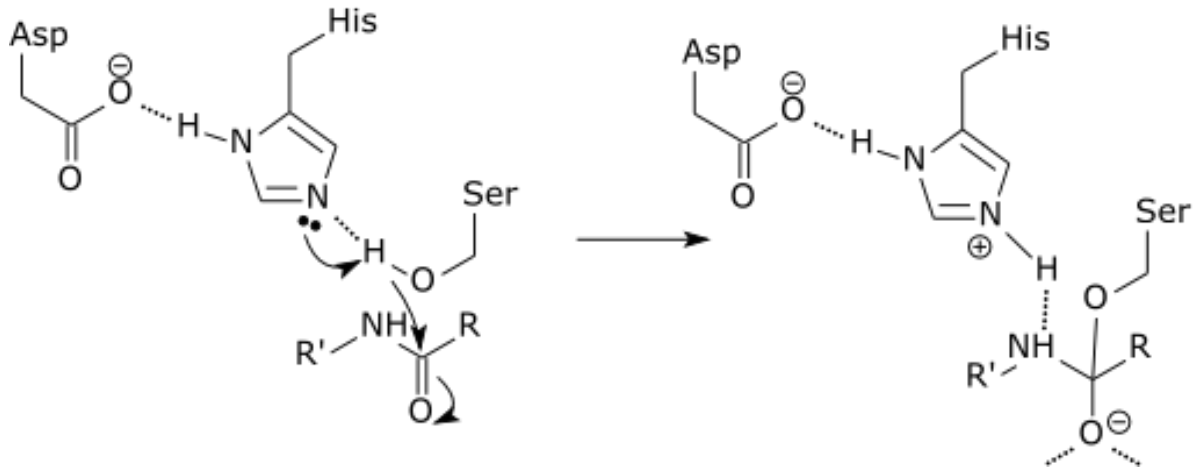

Figure 1: Reaction Mechanism for Serine Proteases.

In order to narrow down which specific neurons would be investigated, gonadotropin releasing hormone (GnRH) neurons were selected for its easy accessibility of the cell cluster making it a good candidate to study what effects serine proteases and their inhibitors have on the migratory process. The research was done in an embryonic chick model because this coherent cell cluster migrates along the olfactory nerve before entering the forebrain and provides easy accession of these migrating cells.

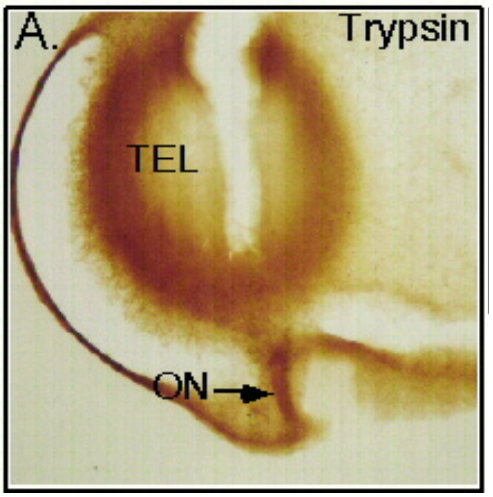

Figure 2: GnRH Neurons Enter Forebrain by Olfactory Nerve (ON).

Figure 2 above shows the pathway that GnRH neurons take to travel into the forebrain, specifically the telencephalon signified by TEL in figure above [1].

There are many factors that influence neuronal migration but a key factor is the expression of the ECM that contribute to the bundles of nerves that axons will travel on [3]. The author suspect that serine proteases and their inhibitors might be involved in changing the quality of the surrounding ECM, by chemoattraction of signaling cues or activating/inactivating target proteases, and play a major role in their migratory process. The serine protease inhibitors inactivate target proteases by binding to them and forming a complex. One member of this family, nexin-1(PN-1), modulates neuronal migration and has been shown to slow granule cell movement from external to internal layers of cerebellar slice cultures by previous studies [4]. It has been shown that the inhibition and stimulation of migration may be balanced due to effects of serine protease inhibitors and serine proteases, respectively. For example, a serine protease called plasmin accelerates migration of neuroblastoma cells, whereas aprotinin, a serine protease inhibitor, decreases migration of the population to the same degree [3]. The authors then conducted a series of experiments to test this hypothesis by examining *in vivo* neuronal migration of chick GnRH neurons during embryogenesis. Thus, by understanding how the balance of serine protease and serine protease inhibitor activity works, one can further understand how these changes affect neuronal migration in development.

In order to test the role that serine proteases and their inhibitors had, the authors used protein soaked beads and inserted them in various locations of interest, such as the placode epithelium. By noticing that the GnRH distribution on the side contralateral to the bead and that of the control (a PB coated bead) were not significantly different, the authors used the contralateral side as a control. Therefore, GnRH distribution ipsilateral to the bead was compared to that of the contralateral side. They were then able to identify where the neurons migrated by staining, analyzing the immunoreactivity, and narrowing down and testing at specific stages to determine what happens at developmental milestones [1].

This research revolves around serine proteases and their inhibitors. Thus, it is imperative to discuss what these specific enzymes are and how they function. Serine proteases are enzymes that cleave peptide bonds in proteins [5]. The specific amino acid serine serves as the residue that is ultimately attached to the enzymes active site. The specific structure of serine proteases can be subcategorized into subtilisin like structure and chymotrypsin like structure. Chymotrypsin is a digestive enzyme produced in the pancreas. It enters the small intestine in its inactive form and becomes activated there to facilitate digestion. Subtilisin follows the same catalytic mechanism as chymotrypsin, which makes it

classified as a serine protease. However, subtilisin is structurally very different from chymotrypsin. Its distinct amino acid sequence from chymotrypsin provides it with a globular structure consisting of several alpha helices and a large beta sheet. While the digestive role of serine proteases is not particularly of interest for our purposes, it demonstrates the dynamic role of these proteins in contributing to various physiological processes. Effectively, the multi-faceted nature of serine proteases makes them a significant target for exploration. Furthermore, the differential sub-structures of serine proteases can possibly distinguish the specific role the proteins play in neural development. Serine protease inhibitors interfere with the catalytic mechanism of serine proteases, by altering serine. Biologically these inhibitors have been known to be involved in the bodies' response to injury through various processes including coagulation, inflammation and tissue repair [6]. This involvement in healing mechanisms makes these molecules captivating proteins to study, as one can explore if they will carry forward this function into a different physiological realm in terms of neural development.

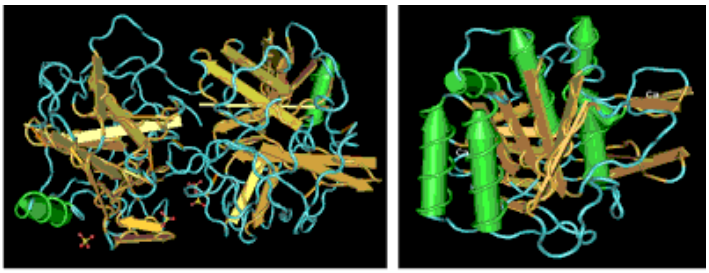

Figure 3: Chymotrypsin and Subtilisin are Structurally Different Classes of Serine Proteases.

Figure 3 above shows a structural comparison between chymotrypsin and subtilisin. Chymotrypsin has more β-sheets but fewer α-helices than subtilisin. Brown arrows = β-sheets Green arrows = α-helices [7].

Effectively, serine proteases and their inhibitors have already been found to play vital roles in other significant biochemical mechanisms of the body [8]. One area where their role is not known is in neural migration, which is also a significant developmental biological event necessary for the survival of an organism. The author would like to explore the critical roles of proteolysis in this neural migration in a particular set of neurons, the chick GnRH neurons. Therefore, would like to conduct experiments exploring the role of these proteases and their inhibitors in critical developmental time points. By applying the proteases and the inhibitors over the developmental stages of 21 to 29 days, it is possible to observe possible alterations to particular migration patterns. These experiments are noteworthy as they are the first to explore the role of these proteins in neural migration in an *in vivo* model. Successful investigation of proteolysis in GnRH neurons can lay the groundworks for exploring the role proteolysis plays in the olfactory nerve, which can be explored using other neural markers.

Preliminary Data

Neural migration is an important aspect in embryonic development of many organisms [9]. It is imperative for proper neuron growth to occur so that the necessary connections and neural networks can be established. If neurons do not migrate to their appropriate locations, then there are abnormal neural connections, which can potentially have deleterious effects on a person's body. Schizencephaly, neuronal heterotopias, and micropolygyria are all neuronal migration disorders (NMD) [10]. Neurogenesis is an extremely complex task that is dependent on multiple highly specific events for proper neural formation such as cell proliferation, migration, and differentiation [11]. The site of neural birth, however, is not always the site of neural function. Consequently, many neurons need to migrate from their origination point to their site of function within the body. Therefore, it is important for not only neurogenesis to occur within embryonic development, but also for neural migration of these neurons to also occur. Mechanisms that aid in the regulation of neuronal migration remain largely unknown but there are various factors that have influence in this temporal and spatial control mechanism [12]. Likely candidates that play roles in this modulation are target site release factors, components of the migratory neurons themselves, and extracellular matrix cues.

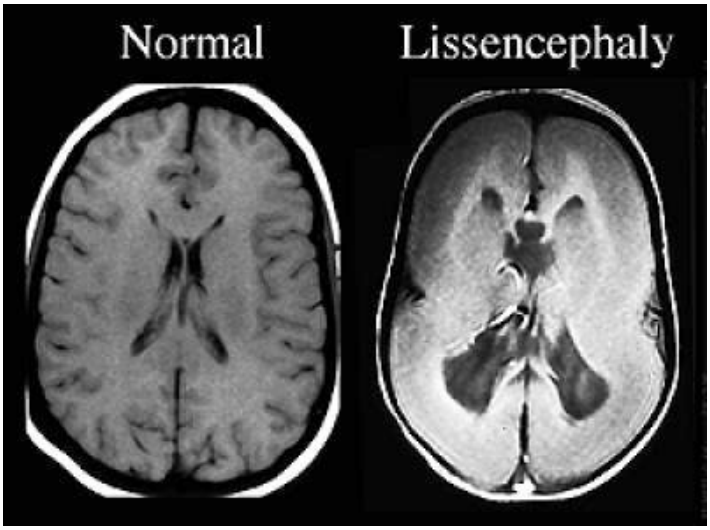

Figure 4: NMD's have Deleterious Effects on Normal Brain Development.

Figure 4 above juxtaposes a normal person's MRI brain scan next to a patient that has lissencephaly. Lissencephaly is a NMD that is the result of abnormal neuronal migration during early gestation, sooner than 30 weeks, that results in a brain without the formation of gyri or sulci, which are the folds and grooves of the brain, respectively [13].

A likely component regulating neuronal migration is the selective spatio-temporal expression of the extracellular matrix. These molecules eventually help contribute to the networks that neurons utilize to propagate their signals. There are some proteins that can alter the quality of this path, by sending out various chemoattraction signaling cues or by activating or inactivating other proteins that contribute to this highway, such as serine proteases and their

inhibitors. By altering these neural "highways," serine proteases can play a pivotal role in GnRH's neuronal migratory process. Serine protease inhibitors, such as nexin-1 (PN-1), act by binding and permanently inactivating target proteases [8]. Serine protease inhibitors that inactivate the protease permanently deactivate the serine hydroxyl group or any other serine residues in the enzymes active site through an esterification process [5]. Previous studies have shown that PN-1 had the unique ability to stimulate neurite outgrowth but it has also been shown that PN-1 modulates neuronal migration as well [4].

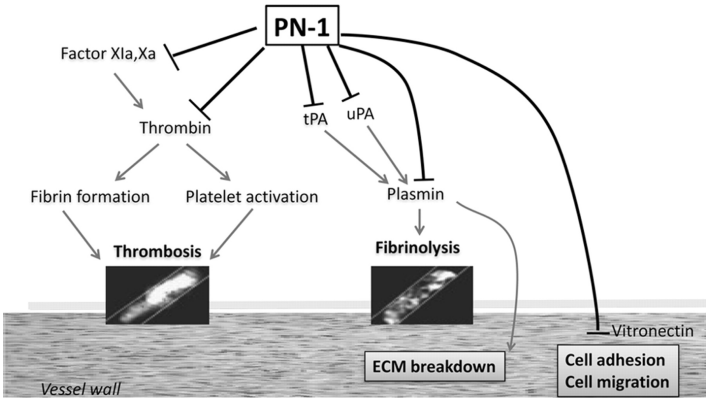

Figure 5: PN-1 affects Quality of Neuronal Highway by Regulating Serine Protease Target Expression.

Figure 5 above shows how PN-1 can modulate the expression of various serine protease targets, such as thrombin, to affect downstream actions, such as fibrin formation in the case of thrombin, in order to change the quality of highly traveled neuronal routes [14].

The inhibition of the migratory mechanism that serine protease inhibitors stimulate is antagonized by neural migration stimulation via serine proteases. Furthermore, there exists a delicate balance between serine proteases and their inhibitors to modulate migration of neurons within development. For example, plasmin, a serine protease, accelerates migration of neuroblastoma cells by a factor of five [3]; however, the *in vitro* addition of aprotinin, a serine protease inhibitor, decreases the migratory population by the same magnitude [15]. Thus, it is easy to see how the balance exists between the antagonistically competing proteins that change the fate of neuronal migration.

This hypothesis was tested *in vivo* by investigating neuronal migration of chick GnRH neurons during embryogenesis. The model organism that they have selected was the embryonic chick. Chick GnRH neurons are differentiated in the olfactory epithelium and migrate along the olfactory nerve before entering the forebrain. The reason why the embryonic chicks were selected was that the neural cell clusters could be easily accessed and analyzed for their migratory processes [1]. Understanding what affects neuronal migration will help to further understand how these mechanisms act on various other neuronal clusters, such as FSH neurons or LH neurons as they have been known to associate with GnRH neurons, and can expand their implications into the medical field, as an injury to the function site of a neuron might not necessarily perturb its function due to it necessary migration to that area.

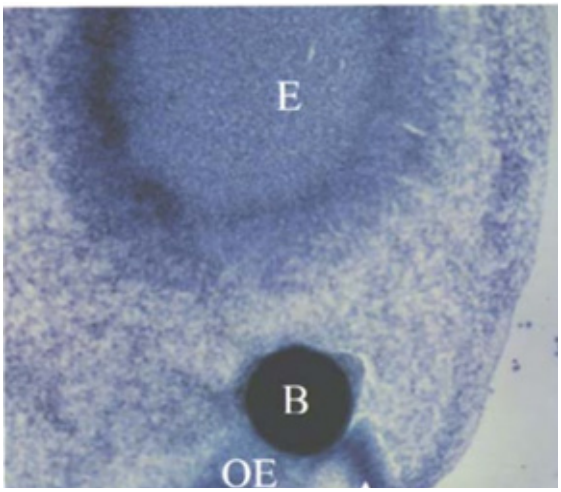

Figure 6: Strategic Locations of Bead Implantation in Embryonic Chick.

The PB bead was inserted at the olfactory placode, which is located just deep to the olfactory epithelium. The PB bead did not disrupt any normal morphology of the tissues. This is also a sagittal cut of stage 21 embryos whose tissue was stained with cresyl violet. E = eye B = bead OE = olfactory epithelium [1].

The site of origin for these cells is in the olfactory placode and the migratory route is alongside the olfactory nerve before entering the forebrain. Previous studies have demonstrated that olfactory axons emerge from olfactory epithelium at late stage 18 and are joined by GnRH neurons at stage 21 days [16]. A protease or its inhibitor was applied by coating the beads and inserting at the olfactory placode. The experiments were divided to test two specific questions. The first tested whether inserting the bead into their desired locations would disrupt initial exit of GnRH neurons from the olfactory epithelium (OE) or affect their rate of migration along olfactory nerve (ON) and into forebrain. The second experiment tested whether GnRH neurons exited the OE independent of the effects of proteolysis on the maturation of the ON. These are the first experiments to demonstrate what roles proteolysis and its inhibition have on GnRH cellular migration.

Since subsequent experiments required the insertion of a foreign object, a protein coated bead, tests were done to determine what effect this introduction would have on GnRH distribution. The control embryos received a protein based (PB) coated bead and the GnRH neurons were counted on both the ipsilateral and contralateral side of the bead. The results indicated that there was no significant effect of the bead insertion on GnRH distributions [1]. This is a critical finding because a confounding variable could have been that the physical act of inserting a bead within the embryo might have disrupted the GnRH neuronal distributions. It was also determined that GnRH distributions of the contralateral

side of the protein coated bead were not significantly different from the PB control beads and as such, the contralateral side of the bead served as the control for the experiment.

In order to understand how serine protease inhibitors, affect the rate of neuronal migration, a specific serine protease inhibitor, PN-1, was examined. PN-1, has four main target proteases: urokinase plasminogenic activator (uPA), tissue type plasminogenic activator (tPA), thrombin, and trypsin, as can be seen in figure 5 [1]. Previous studies have shown that uPA and trypsin play roles in the neuronal migratory process [17]. Consequently, both uPA and trypsin were tested in further experiments, due to their involvement while not testing the other two target proteases and the treated ipsilateral side was compared to the untreated contralateral side of the bead. Implantation of trypsin-coated beads into stage 17 days embryos had no significant impact on either timing of nerve development or biochemical differentiation of axons. However, the distribution of GnRH neurons was altered, as in normal conditions; GnRH neurons are located in the telencephalon by stage 25 days. However, in the experimental conditions, the inclusion of trypsin, GnRH neurons were observed crossing the brain/nerve junction at stage 21 days. This is indicative of acceleration of this event. Furthermore, these cells continued along their migratory route entering their normal target areas, the septal and preoptic regions. Application of uPA beads had no effect on axonogenesis or differentiation and the GnRH distribution was not altered [1]. The authors next turned their attention to the extracellular matrix and hypothesized that there are signal cues given off that regulate the balance of serine proteases and their inhibitors.

Modifying the extracellular matrix environment by modulating the protease/inhibitor balance regulates neuronal migration. The authors carried out other experiments to test this claim and determined that inhibition of endogenous trypsin activity by PN-1 resulted in fewer GnRH neurons leaving the OE [1]. Thus, decreasing the number of neurons entering the brain. Conversely, increasing trypsin activity promoted neuronal crossing of these boundary zones. The authors hypothesized that the serine/inhibitor pair act by changing the extracellular matrix through which GnRH neurons migrate. By inhibiting local proteolysis, PN-1 produces a non-conducive environment at these points and promoting local proteolysis, by trypsin, produces a permissive migratory environment for the neurons [1].

The discovery that mammalian PN-1 changes neuronal migration in avian species is highly suggestive of the fact that the presence of endogenous serine protease activity exists within the region. The authors tested the biochemical characteristic of this endogenous activity by investigating non-neurally derived trypsin inhibitors. As such, soybean trypsin inhibitor was tested and had no effect on GnRH migration. A possible reason for this might have been that there are many differences in binding affinity between soybean trypsin inhibitor and PN-1 for endogenous trypsin-like molecules. Trypsin was then examined because it results in global acceleration of GnRH neuronal migration out of the OE and into the brain. In the chick, trypsin immunoreactivity was present throughout the head in addition to the ON. The ubiquitous distribution is indicative of the specificity that the protease/inhibitor system may produce by an avian equivalent of PN-1. Conversely, uPA-treated embryos had no effect on GnRH distributions and no uPA immunoreactivity [1]. The authors further concluded that uPA might function in synaptogenesis and remodeling as opposed to cell migration. Due to PN-1's ability to stimulate neurite growth, the authors then posited the question of whether PN-1 could change the timing of the formation of the ON and how this might affect distribution of GnRH neuronal migration.

To test the hypothesis that it does affect ON formation, the authors inserted beads into the olfactory placode of stage 17 days embryos just before ON axonogenesis. The authors found that PN-1 had no effect in accelerating neurite outgrowth but did accelerate biochemical differentiation markers of the ON [1]. This finding suggests that olfactory neurons respond directly to PN-1 or to changes in local environment caused by PN-1. Even though there was accelerated maturation of the ON, GnRH neuron distribution was not altered by PN-1. Furthermore, PN-1 had no effect of the timing of the nerve's ensheathment by glia [1]. This finding suggests that glial migration is independent of any matrix changes induced by PN-1.

Taken together, these findings indicate that the ability to initiate migration lies inherently within the neuron itself such that changes of the extracellular matrix composition are irrelevant to the timing of the initial event during stage 17-21 days embryos. The authors came to this conclusion by the observation that the addition of trypsin or PN-1 does not show significant results because GnRH neurons exit the OE at stage 21 days as in controls. The authors found that neurons enter CNS earlier in trypsin-treated embryos indicating that continuous movement may be a cell autonomous event with trypsin-induced proteolysis. This shows that GnRH neuron's ability to migrate across glia boundaries and through the brain to its target site is independent of further maturation of the neuron, the ON, or the brain.

In light of all the experiments that have been conducted, the next logical question should be directed at the differing and inherent mechanisms that are present in the neuron that allow it to migrate independently of extracellular matrix cues during specific stages 17-21 days. Further investigation is necessary to determine what effects changing the type of serine protease will have on neuronal migration, such as comparing trypsin and chymotrypsin and their respective inhibitors [6].

Specific Aims

In early embryonic development, neural migration leads to neuron growth and connections within neural networks. Macromolecules that have been shown to have a role in this neural migration are serine proteases and their inhibitors. The role of serine proteases in neural migration can be explored by inserting a protein coated bead into the olfactory placode of a chick embryo. This is a good model for investigating this phenomenon, because the neural cell clusters of these chicks can be simply analyzed before and after

manipulation with serine proteases and serine protease inhibitors [1]. This system of neural migration is a very important process for the viability of an organism, as disturbances in the development of the nervous system can lead to severely debilitating results. Furthermore, while much is known about the function of serine protease and their inhibitors, these proteins have not been explored in relation to nervous system development.

A question we are interested in exploring involves looking at whether the specific type of serine protease has any distinguishing effect in its modulation of neural migration. There are two known structures of serine proteases trypsin like and chymotrypsin like. It has been found that inherent mechanisms involving the neurons themselves leads to migration initiation, as opposed to ECM cues. It is also known that serine proteases have a role in the initiation of migration. However, the specifics of the serine proteases involved in this initiation is not known. This question can be explored by implanting a bead with a trypsin class serine protease protein in one embryonic chick and doing the same in another chick embryo instead with a chymotrypsin protease coated bead, as can be seen in figure 6. Both beads will be inserted in the olfactory placode of stage 17 days embryos. The rate of exit from the olfactory epithelium and the rate of migration along the olfactory nerve to the forebrain will be measured after the insertion of both types of beads. These rates will be measured on the ipsilateral side of the embryo with the contralateral side serving as a control, because this was established by prior experiments [1]. Furthermore, as another control, the baseline rate of migration will be measured in a chick embryo that has a bead implanted with no protein coat to ensure that differences in neural migration are not due to intrinsic differences between the embryos themselves or in the presence of the implanted bead. The significance or lack of significance between the neural migrations caused by these two types of serine proteases will inform us about whether the particular serine protease structure has a developmental effect on the chick embryo. These findings can provide significant information about the mechanism of these proteins in relation to nervous system development, and whether the mechanism is dependent upon protease structure.

Specific Aim Question

Future research involves looking at whether the specific type of serine protease has any distinguishing effect in its modulation of neural migration because migration errors in GnRH neurons can halt this migration in embryonic organisms. There are two known structures of serine proteases, trypsin like and chymotrypsin like [18]. It has been found that inherent mechanisms involving the neurons themselves leads to migration initiation, as opposed to extracellular matrix cues [1]. This fact was established by the finding of our experiment with the extracellular matrix cue and serine protease inhibitor PN-1. PN-1 did not significantly alter the stage at which GnRH neurons exit the olfactory epithelium when compared to the control [1]. Nevertheless, because it is known that serine proteases have a role in the initiation of migration; other mechanisms should be explored [1]. However, the exact serine proteases involved in this initiation is not known. In order to test whether or not different types of serine proteases affect neuronal migration, we will implant experimental beads coated with trypsin and chymotrypsin into the olfactory placode of the embryonic chick at stage 17 days. In order to distinguish if whether or not the type of serine protease inhibitor modulates neuronal migration, the beads will be coated with two different serine protease inhibitor types and surgically implanted at the placode within the chick. We expect to find that there would be a difference between the serine protease inhibitor types. While we are unsure which inhibitor type would cause the greatest modulation of neuronal migration, the results would inform us as to whether structural differences play a role in this process within development of embryonic chick.

Experimental Plan

The embryonic chick model will be utilized in answering this question due to its ease in accessing and monitoring the GnRH cell clusters and because the importance of neuronal stages is well known in this model. The chick will be implanted with a bead coated with a trypsin class serine protease protein, while another chick will be implanted with a bead coated with a chymotrypsin protease, as in figure 6. The specific neurons that will be observed after the bead insertion are the GnRH neurons. These specific neurons release reproductive hormones such as luteinizing hormone (LH) and follicle stimulating hormone (FSH). As a result, these neurons have a great developmental role in transitioning an organism from being a juvenile to an adult [19].

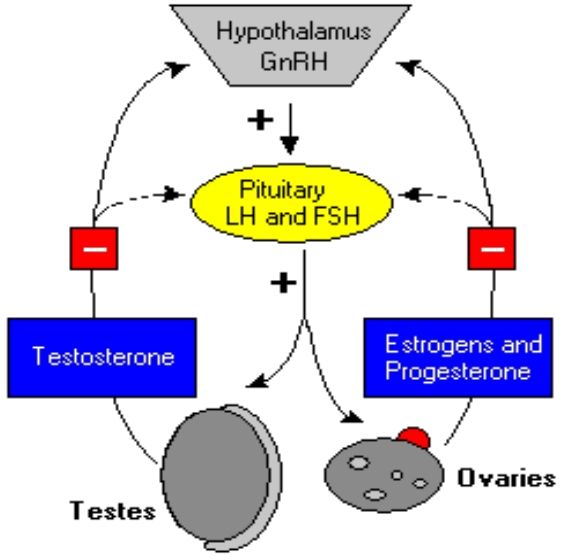

Figure 7: GnRH Neurons Regulate FSH and LH Neuronal Expression.

Figure 7 above shows how the GnRH neurons affect the release of the reproductive hormones FSH and LH. Thus, the migration of GnRH neurons are critical for the proper development of an organism, as these neurons are responsible for the transition between the juvenile to adult stage [20].

The insertion of these beads is hypothesized to affect the migration of the GnRH neurons from the olfactory epithelium based on its specific coating. Both beads will be inserted in the olfactory placode of stage 17 days embryos. Starting at the same stage will

serve as the basis for the comparison of these two chick embryos. The beads will be inserted at this specific stage because stages 17-21 days have been found to be critical to the development of the chick embryo [1]. The rate of exit of the GnRH neurons from the olfactory epithelium and the rate of migration of these same neurons along the olfactory nerve to the forebrain will be measured after the insertion of both types of beads. These rates will be measured on the ipsilateral side of the embryo with the contralateral side serving as a control, because this was established by prior experiments [1]. Our previous research has already shown that the insertion of the bead itself does not alter the neuron migration rates [1]. As another control, the baseline rate of migration will be measured in a chick embryo that has no bead inserted to ensure that differences in neural migration are not due to intrinsic differences between the embryos themselves. In this experiment, we expect to see a significant difference in rate of migration of the GnRH neurons for the two beads coated with different classes of serine proteases. While we are not sure which class of serine proteases will have a greater effect on the GnRH neuron migration, the significance between the neural migrations caused by these two types of serine proteases will inform us about whether the particular serine protease structure has a developmental effect on the chick embryo. Specifically, it will tell us which structure of serine proteases is most beneficial for facilitating the neuronal migration of GnRH neurons. Essentially, these findings can provide significant information about the mechanism of these proteins in relation to nervous system development, and whether the mechanism is dependent upon protease structure. If this experiment is successful, we can further expand this procedure by applying the same steps stated above and observing the migration of a different subset of neurons. Doing so will allow us to determine the universality of our findings about the effect of serine protease structure and its effects on all neuronal migration as opposed to just GnRH neurons. If no significant difference were found between the average rates of migration of GnRH for the two classes of proteases, we would ascertain that serine protease structure is not an essential factor altering the migration of these neurons. In that case, we would have to look towards another mechanism that may play a role in this system. Statistically, the results of this experiment can be analyzed using a t-test to compare the average migration rate of the GnRH neurons for the two classes of serine proteases; this statistical test will allow us to see if there is a significant difference between the average migration rates for these two groups [21].

Taken together, this experiment provides a vast array of knowledge regarding these proteins and their relation to development. We will be able to determine if the structure of the serine protease inhibitor plays a role in modulating the rate of neuronal migration. This knowledge can potentially lead to therapies that can provide ameliorative care for patients suffering with NMD. If such a connection between structure of serine protease inhibitors and their function is found, subsequent experiments can provide information about how to treat specific types of ailments and what serine protease would be best fit to do so.

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