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**Research Proposal BNFO 300: PTK7 Domain Involvement in Planar Cell Polarity Pathway Regulation of Zebrafish CoPA Neurons Axon Pathfinding**

**Introduction**:

 As humans what separates us from the rest of the animal kingdom is our ability of higher cognitive functioning. The human nervous system is a very complex, and there is much to learn about how it works. One area which is of interest when trying to understand how the human brain works is how axon path finding works during neuronal development in early stages of life. Learning how neurons make connections and why they make the specific connections they do is crucial for understanding how to map the human brain.

Zebrafish are a model organism, which have been the subject of scientific inquiry for some time. The development of neurons has always been an area of interest when trying to understand the human brain and zebrafish are an excellent media to learn about the human nervous system because of the conserved cellular mechanisms between the two species. The CNS of early zebrafish is an especially interesting environment. A specialized zebrafish TBX16 has been engineered to further the research of neuron development in these fish. This TBX16 zebrafish is engineered to express Green Fluorescent Protein (GFP) in Commissural Primary Ascending neurons, CoPA; these secondary interneurons are involved in a touch/pain retraction mechanism (Ruvinsky et al 1998). Commissural Primary Ascending neurons are cells which sit in the spinal cord and project axons to the CNS/Brain. Each Commissural neuron sits in a different level of the spinal cord, and during development, axonal migration is regulated by a cell signaling pathway called the Planar Cell Polarity (PCP) pathway. In zebrafish, primary commissural ascending neurons (CoPA), are interneurons which, during development, project their axons ventrally and crosses the midline to grows dorsally; where it then turns and ascends the spinal cord anteriorly towards the CNS/Brain (Hjorth & Key 2002; Bernhardt et al 1990). Each CoPA neuron has a target cell in the CNS, and uses the Wnt signaling molecule gradients to mediate theses axons pathfinding and growth. Wnt is the ligand which regulates PCP pathway in copa neurons of zebrafish; the concentration gradient of Wnt directs axon migration towards the proper target (Hayes et al 2013).

Planar Cell Polarity (PCP) coordinates the uniform orientation of cell structures and cell movements within the cells plane of tissue (Zallen, 2007). A group of evolutionarily conserved genes affecting PCP in all tissues are often referred to as the “core” PCP genes; these genes include the proteins Frizzled (Fz), Flamingo (Fmi), Van Gogh (VangL), Disheveled (Dsh), Prickle (Pk) and Diego (Dgo) (Vladar et al 2009). The main action in this pathway, which mediates cell processes and cytoskeletal structure, is Frizzled binding to Wnt (Kohn and Moon, 2005; Ciruna et al, 2006). Wnt will bind to Fz extracellularly, which will change Fz’s affinity to Dsh and cause it to interact with the intracellular proteins. Dsh then goes on to activate a secondary messenger system which affects JNK (Boutros et al 1999); JNK is a mediator of cell movement via actin filament structure, so once it is activated by Dsh, it goes on to affect the actin cytoskeleton and change shape/movement (Yamanaka et al 2002). PCP requires Fz activation and subsequent membrane localization of Dsh; Fz activation occurs through the binding to Wnt (Axelrod et al 1998).

When observing the functional and morphological deficits of PCP core gene mutant vertebrates, you see very similar deficits between different genes. In vangL mutant zebrafish, embryos demonstrated neural tube morphological defects (Hayes et al 2013). PTK7 was found to be a novel regulator in the planar cell polarity pathway (Lu et al 2004; Hayes et al 2013); in the experiment described in Lu et al 2004 the research team was demonstrated that mice with mutant ptk7 genes displayed a severe form of neural tube defect (NTD). Hayes et al in 2013 reported that ptk7 mutant zebrafish embryos demonstrated neural tube morphological defects similar to those observed with vangL mutant zebrafish; which is a known core PCP signaling gene (Hayes et al 2013). So the same defects were seen in ptk7 mutant zebrafish and in vangL mutant zebrafish, suggesting the two are involved in the same signaling pathway, Planar Cell Polarity (Lu et al 2004). PCP core genes are required for axon pathfinding in Copa neurons in zebrafish (Simon Sun. 2014); so CoPA neurons in zebrafish which express a mutant version of any PCP core genes will not all turn anterior after crossing over the midline (wild type). These CoPA neurons will express a randomization of A-P guidance; a 50:50 distribution of axons travelling anterior and posterior was observed in studies using other PCP core gene mutants (Simon Son. 2014).

PTK-7 is a protein comprised of a single polypeptide chain with one transmembrane domain. The extracellular domain is composed of several immunoglobulin like domains. The transmembrane domain is a combination of hydrophobic amino acids which come together to form an alpha-helix in the membrane. The intracellular domain of this protein contains a conserved tyrosine motif (Hayes et al 2013). It is not known which of these domains, if any all, are involved in regulating the PCP pathway in zebrafish CoPA neurons. In order to determine which domains of ptk7 are required in this pathway, Hayes et al 2013 tested the ability of deletion and substitution mutants to rescue wnt8 overexpression defects in embryonic zebrafish. It has been seen that ptk7ΔICD (deleted intracellular domain) and ptk7EgfrTM (exchanged transmembrane domain) were able to rescue the wnt8 induced defects to the same extent as full length ptk7. It was also observed that Ptk7ΔECD (deleted extracellular domain) was not able to rescue wnt8 overexpression defects. This suggested that the extracellular domain is most likely involved in regulating the PCP pathway in zebrafish CoPA neurons (Hayes et al 2013). This domain could be involved in co-receptor activity with Frizzled and Wnt ligand.

**Experiment:**

**PTK7 Rescue Experiment:**

In this experiment we will be testing the ability of modified PTK7 genes ability to rescue ptk7-deficient mutant zebrafish copa neuron axon migration deficits. The different modified versions of PTK7 will be ptk7ΔICD (deleted intracellular domain), ptk7ΔECD (deleted extracellular domain) and ptk7EgfrTM (exchanged transmembrane domain) and full length PTK7. If the anterior/posterior randomized selection of these CoPA neurons can be rescued to result in mostly anterior/wild type (not always 100% in real life) without one of PTK7’s three domains, then that domain can be concluded to not be required for regulating the PCP pathway in zebrafish CoPA neuron axon migration.

PTK7-deficient mutant zebrafish are generated from TBX16 zebrafish by manipulating Zinc Finger Nucleases to knock out the PTK7 gene. The mutant PTK7 zebrafish would be microinjected with a specially engineered plasmid containing the cDNA for experimentally modified PTK7 protein (Fig 2), and isolated Tol2 transposon mRNA at the one cell stage of development (Kawakami 2007). The plasmid will contain a full version of ptk7 or a modified version of ptk7; to express either deleted intracellular/extracellular domains or substituted transmembrane domain. Fig 1 gives an example of what each experimental groups protein will look like. The plasmid will also encode a heat shock promotor (HSP-70), a poly-A tail, and M-Cherry tag; all of which is in between two Tol2 transposase sites. The heat shock promotor, HSP-70, will allow for the controlled activation of the inserted genetic information (Holloran et al 2000). Tol2 is a transposon which has been shown to be a versatile method of gene transfer in vertebrates; it is very effective and can transfer genes of up to 11kb with minimal error (Kawakami 2007). This method of gene transfer will result in a mosaic model of cells in the organism which express the genetic information of interest. In this experiment, zebrafish CoPA neurons which express plasmid DNA will also express M-Cherry and GFP; so the combination of both is what will be used to identify CoPA which effect experimental outcome.

*(Hayes et al 2013) Fig 1.*

 *Diagram showing the structure of each experimental PTK7 protein being used in this experiment.*



 *Fig 2. Diagram showing the structure of plasmid generated by experimental procedure.*

 Once mRNA and plasmid DNA are microinjected in the one cell stage embryos of ptk7 mutant zebrafish, the mRNA takes some time to be translated and folded into fully functional transposon protein, Tol2. Once it is formed, this transposon will randomly insert the genetic information in between the two Tol2 transposase sites into the genome of the mutant zebrafish (Kawakami 2007). By the time the mRNA is folded and plasmid genes are able to be incorporated, the organism will be at around the 1064 cell stage, so not all of the cells will express the modified version of the ptk7 protein. A mosaic model of cells which express the plasmid DNA will be observed (Simon Son. 2014).

**Generation of PTK7 Mutant Zebrafish:**

You first start off with a mutant zebrafish, TBX16 zebrafish. These zebrafish are genetically engineered to express Green Fluorescent Protein (GFP) in Copa neurons (Ruvinsky et al 1998). The next step is to turn the TBX16 zebrafish into a mutant PTK7 zebrafish through gene knockout; meaning that is will not have a functional version of the ptk7 protein in its genome. Zinc Finger Nucleases (ZFNs) can be used to knockout targeted genes, such as ptk7, in zebrafish (Meng et al 2008). ZFNs are chimeric proteins which act as heterodimers to cleave a desired target site; the ZFNs can be engineered to recognize a target DNA sequence of interest, such as ptk7. ZFNs can be introduced to zebrafish by injecting mRNA encoding each ZFN monomer at the one cell stage of the F1 generation. These ZFNs will be engineered to target ptk7. Upon binding to the DNA of ptk7, it will cause a double strand break in the DNA; which will be repaired, but not without irreparable damage in the form of a deletion or substitution mutation. Once grown, some zebrafish will be wild type and some will have the knocked out ptk7 gene. Those which express the knockout will be selected and crossed to bread a generation of all mutant ptk7 zebrafish.

**Generation of Plasmid:**

Next plasmids will need to be constructed which contains modified versions of ptk7. This first starts with isolating and amplifying coding DNA (cDNA) for ptk7 protein in zebrafish using PCR. Specially designed primers will be used to start and stop the replication of the protein at certain sites to allow for substitution and deletion of the protein. These primers will also be engineered to include attb1, attb2 and M-cherry (fluorescent dye) sites on each end of each DNA piece during PCR. Once these modified, isolated and amplified cDNA fragments are complete for experimental set-up, they will be inserted into an entry vector through enzymatic recombination (Hartley et al, 2000; Walhout et al. 2000). The plasmid containing the ptk7 cDNA of interest will have the DNA in between the two sequences, attB1 and attB2. The DNA in between these sequences will be switched with the DNA in the target vector in-between attP1 and attP2 sites via recombination by BP clonase crossing over attB1/attB2 with attP1/attP2 to create new attL1 and attL2 sites (Villefranc et al 2007); the DNA inside the target vector is a gene which encodes a protein which will kill the the bacteria which expresses it, ccdB (Hartley et al 2000). The entry vector plasmid contains a kanamycin resistance gene, which when grown on a kanamycin agar plate, will ensure that only bacteria expressing this resistance will survive. If some cells were not involved in the recombination BP reaction, then they will already contain genetic information which will cause cell death, ccdB (Villefranc et al 2007).

Once the DNA of interest is in the entry vector it can then be transferred over to a destination vector. This destination vector will be engineered to have a heat shock promoter (HSP-70), R1 site, ccdB gene, R2 sites, and poly A tail all in between two tol2 transposase sites (in that order). The destination vector plasmid will be carrying a gene for ampicillin resistance. This will ensure that only bacteria expressing recombined plasmids will survive in an ampicillin/agar growth plate. LR Clonase will perform the recombination of DNA between attL1 and attL2 with DNA between R1 and R2 sites. Once this LR reaction is completed and the bacteria allowed to grow, there should only be bacteria which contain the plasmid with the DNA sequence of interest inserted into the target vector which expresses ampicillin resistance (Villefranc et al 2007).

Discussion:

Copa neurons are second order sensory neurons in zebrafish which are part of a touch/pain evoked retraction mechanism. In the transgenic zebrafish generated for this experiment, Green Fluorescent Protein (GFP) is expressed in all CoPA neurons in the organism. These neurons during development in zebrafish, without PCP gene defects, will send their axons up the anterolateral track up to the brain (Simon Sun. 2014). This means the axon must first cross the midline of the spinal cord, then change direction to go up the spinal cord towards the brain (Anterior). In zebrafish which express mutant or non-functional versions of PCP core genes, including ptk7, these CoPA neurons will tend to randomly choose to turn anterior or posterior in the spinal cord after sending their axons cross the midline of the spinal cord; randomization of A-P guidance (Simon Son. 2014).

In this experiment, the inserted genomic information includes an M-Cherry fluorescent protein, so any CoPA neuron which expresses the plasmid DNA will fluoresce both red and green (M-Cherry & GFP). When analyzing the CoPA neurons of zebrafish in this rescue experiment, the neurons which express GFP and M-Cherry are the only ones to consider when comparing how many axons travel anterior vs posterior after crossing the midline. In the experimental group whose plasmid includes full length ptk7, there should theoretically be 100% of copa neuron who express both GFP and M-Cherry whose axons travel anterior after crossing the midline. If the A-P guidance can be rescued using a version of PTK7 with a deleted or substituted domain, then that domain must not be required in the regulation of this pathway.

 This experiment has some downsides and limitations that should be addressed. One limitation to this experiment is the mosaic model of trans-genesis in the zebrafish seen from using this method of genetic modification. The transposon mRNA takes time to be translated and processed into the functional protein. So microinjection at the single cell stage will result in only a distribution of cells which incorporate the plasmid DNA into their genome. So a mosaic of model of cells which express the plasmid DNA is observed. This can be problematic when attempting to look at the effects of a specific protein on a specific cell type. If fortunate, at least one copa neuron will express the plasmid DNA in a single zebrafish; in some zebrafish there will be no expression of plasmid DNA in the desired cell type.

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