IDENTIFYING NEURON CLUSTERS CONTROLLING MOVEMENT VIGOR

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Abstract

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Parkinson's disease (PD) is a neurodegenerative disease that progressively decreases dopaminergic function. Lower amounts of dopamine may cause an innate motivational shift that decreases movement vigor when performing difficult tasks. In PD patients, bradykinesia, the slowing of movement, is characteristic of this decrease in vigor. The movement vigor hypothesis proposes that dopamine neurons modulate performance aspects of movement, like speed and persistence. This paper proposes a series experiments utilizing neuron recording techniques in zebrafish that would test the movement vigor hypothesis by determining whether in fact there is a distinct group of dopamine neurons that modulate movement vigor. Core elements of the human dopamine motor system are shared by all vertebrates, so zebrafish are a valid model system for studying dopamine's role in controlling movement vigor. The zebrafish behavior used to study vigor in the proposed experiments is a dive response to a sudden decrease in ambient illumination (dark flash evoked dive). This behavior is remarkably persistent, and its duration, speed and distance will be used to quantify the vigor components of movement. In humans, dopamine deficits affect movement vigor particularly when there is an expectation that the required movement will be difficult. Therefore, zebrafish will be trained to perform easy (short) or difficult (longer) dives, and dopamine neuron activation will be compared across those conditions to identify neurons whose activity

correlates with vigor level. If vigor related neurons are identified, subsequent experiments will test whether they are necessary for normal modulation of movement vigor.

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Parkinson's disease (PD) is one of the most common neurodegenerative diseases and is characterized by motor impairments (Flinn et al., 2008; Matsui et al., 2009; Migliore & Coppedè, 2009; Morales-Garcia et al., 2017). With the numbers of affected people projected to continue rising it is unfortunate that there is still much that is unknow about the etiology of the disease (Migliore & Coppedè, 2009). A loss of dopaminergic function is believed to be the key reason PD patients experience motor impairments (Albanese & Lalli, 2010; Morales-Garcia et al., 2017). PD causes a decrease in the cellular production of striatal dopamine lowering amounts in the vesicles of the synaptic cleft (Sulzer et al., 2016). As the disease advances, a sizeable loss of dopaminergic neurons progressively occurs as cell death continuously increases (Gao et al., 2015). PD pathology demonstrates that this neuronal loss mostly occurs in the pars compacta of the substantia nigra, a deep forebrain nucleus within the basal ganglia (Gao et al., 2015).

Motor impairments appear as but are not limited to tremors, rigidity, dystonia, akinesia, and bradykinesia (Gao et al., 2015; Morales-Garcia et al., 2017; Schilder et al., 2017). PD tremors differ slightly from other types of tremors because they typically occur at rest and disappear with movement (Albanese & Lalli, 2010; Shibasaki, 2011). Rigidity is a stiffness that occurs when trying to move limbs that decreases an individual's range of motion (Malek & Grosset, 2012). Dystonia is categorized as uncontrollable muscle contractions that cause repetitive movements (Albanese & Lalli, 2010). Akinesia is the loss of movement (Schilder et al., 2017). Bradykinesia is the slowing of movements (Mazzoni et al., 2007).

There is an importance to understanding the mechanisms that control movement because a better understanding could ultimately lead to discovering novel treatment options for movement related diseases. Since decreased concentrations of dopaminergic neurons is believed to be the cause for PD motor impairments, it is the key target in the treatment of the disease (Gao et al., 2015).

Unfortunately, dopamine increasing PD drugs are limited in their capabilities and are only able to treat the symptomology of the disease, not cure it (Morales-Garcia et al., 2017). Moreover, these medications can cause negative side effects of their own including involuntary muscle movements called dyskinesia, which occurs when dopamine boosting drugs wears off and dopamine levels become unbalanced. (Malek & Grosset, 2012). There is currently no known cure for PD, only methods to slow its progression and treat its symptoms (Morales-Garcia et al., 2017).

Dopamine neuroanatomy and behavioral function

The function of basal ganglia dopamine neurons is not limited to motor control, and dopamine deficit can disrupt functions other than movement (Albin et al., 1989). Various hypotheses regarding dopamine's functions have been created and disputed over time, including reward seeking, avoidance behaviors, cognitive functions such as memory and attention, affect, anxiety, and motivation (Hikida et al., 2012; Jacob et al, 2010; Mele et al., 2019; Sonne et al., 2020). PD patients are at elevated risk for deficits in these functions, supporting the importance of dopamine for their normal execution (Gepshtein et al., 2014; Jacob et al., 2010; Mazzoni et al., 2007; Turner & Desmurget, 2010). This paper proposes a series of experiments aimed at understanding dopamine's

role in motivation and its relationship to motor function. The sections below will first review dopamine neuroanatomy and behavioral function to provide necessary context for the research proposal. After discussing neuroanatomy, separate sections are presented on dopamine's function in reward seeking and expectancies, cognitive functions, motor control, and motivation.

Anatomy of dopamine pathways in the vertebrate brain

In the centra nervous system (CNS) there are four major dopaminergic pathways that determine dopamine's function, the mesolimbic, nigrostriatal, mesocortical, and tuberoinfundibular pathways (Beaulieu & Gainetdinov, 2011). Dopaminergic regions send projections throughout these various pathways causing a variety of effects when they arrive at their target destinations in the brain (Ko & Strafella, 2012; Yao et al., 2008). Projections begin in the midbrain traveling to the forebrain across pathways with targets that influence different dopamergic actions (Yao et al., 2008). Dopaminergic projections that begin in the ventral tegmental area (VTA) A10 travel through the pathways of the mesocorticolimbic system (Yamaguchi et al., 2011; Yao et al., 2008). The mesocorticolimbic system is comprised of two pathways that influence motor learning, reward, regulate mood, influence motivation, and cognition (Yamaguchi et al., 2011). The mesocortiol pathways sends projections to areas of the cerebral cortex while the mesolimbic pathways projects to the nucleus accumbens in the ventral striatum and other limbic structures (Ko & Strafella, 2012; Yao et al., 2008). The substantia nigra of the midbrain sends projections to the forebrain dorsal striatum through the nigrostriatal pathway (Sonne et al., 2020; Yao et al., 2008). The substantia nigra is comprised of two

subregions including the pars compacta and pars reticulata (Sonne et al., 2020). As previously mentioned, the pars compacta of the substantia nigra is the dopaminergic brain region associated with movement and linked to PD (Gao et al., 2015). The dorsal striatum is comprised of the caudate nucleus and the putamen (Sonne et al., 2020). The tuberoinfundibular pathways originates in the hypothalamus and is responsible for triggering the pituitary gland to secret prolactin (Habibi, 2010). This pathway regulates the endocrine or hormonal function of dopamine. Dopamine's modulation of these various pathways controls movement, influences motivation, reward-seeking, regulates hormone production, along with facilitating learning and memory (Beaulieu & Gainetdinov, 2011).

The molecular regulation of dopamine neurotransmission is equally important to understand as dopamine's anatomical circuits for insight into dopamine's normal functions related disorders. 3-hydroxytyramine (dopamine) is a derivative of the amino acid tyrosine (Carlsson et al., 1957). Tyrosine is converted into Levodopa (L-DOPA), the precursor to dopamine by the enzyme tyrosine hydroxylase (TH) (Carlsson et al., 1957). L-DOPA remains the primary treatment for PD motor symptoms since being first used for it in the 1960's (Abbott, 2010; Naughton et al., 2016). L-DOPA possesses the ability to cross the blood-brain barrier, something that dopamine cannot (Juorio et al., 1993). L-DOPA is then converted into dopamine by another enzyme called DOPA decarboxylase (Carlsson et al., 1957). Carlsson et al. (1957) determined dopamine's role as a neurotransmitter. In attempting to better understand dopamine it is important to identify how it works in the brain. Dopamine can have varying effects on neural circuitry

depending on its concentration and location in the brain (Calabresi et al., 2007; Yao et al., 2008). Dopamine transporters (DAT), vesicular monoamine transporters (VMAT), autoreceptors, and metabolic enzymes are all factors that can influence the variation experienced throughout the various regions of the brain (Bromberg-Martin et al., 2010). DATs transport extracellular dopamine molecules against its concentration gradient from the synaptic cleft back into the presynaptic neuron (Sulzer et al., 2016). VMAT2 provides a means of transportation through proton pumps that energize the transport of dopamine and other monoamines into vesicles for later release (Sulzer et al., 2016). Dopamine autoreceptors provide feedback that decreases the excitability and synthesis of dopamine by inhibiting the opening of Ca+ channels (Sulzer et al., 2016). Enzymes play a role throughout the process of dopamine synthesis and breakdown. Tyrosine hydroxylase is the enzyme that limits dopamine synthesis during the conversion of tyrosine into L-DOPA (Olguín et at., 2015). Monoamine oxidase and catechol-O-methyl transferase are enzymes that breakdown dopamine (Olguín et at., 2015). Dopamine acts on dopamine specific G-protein coupled receptors (Yao et al., 2008). These transmembrane receptors are encoded by dopamine genes (Yao et al., 2008). Dopamine receptors play a critical role by conveying feedback that influences the subsequent release of dopamine, the up or down regulation of dopamine (Sulzer et al., 2016). There are five dopamine receptors types, D1-D5 but they are typically categorized under the D1-like or D2-like receptor class umbrella because of their similarities (Yao et al., 2008). These pairings occur because D1 and D5 are excitatory and D2-D4 are inhibitory (Yao et al., 2008). D1 receptor excitability is due to the stimulation of the alpha subunit of the G protein which

increases cyclic AMP (cAMP) via adenosine cyclase, turning on protein kinase A (PKA) and causing the phosphorylation I1 and DARP-32, the added phosphate group causes the inhibition of protein phosphates 1(PP1) (Arias-Carrión & Pŏppel, 2007; Leslie & Nairn, 2019; Yao et al., 2008). The inhibitory properties of D2 receptors are the product of suppressing adenosine cyclase which prevents the subsequent downstream actions associated with cAMP (Arias-Carrión & Pŏppel, 2007; Yao et al., 2008).

Dopamine function: Reward-seeking and reward expectation.

Dopamine's association with reward-seeking is mostly attributed to its influence on the nucleus accumbens, an area often associated with pleasure (Weiss et al., 2000). Pairing the release of this neurotransmitter with a stimulus is enough to condition a cascade of repeated and desired behaviors through reinforcement (Weiss et al., 2000). For decades, experimenters have utilized this method to test various hypotheses about reward seeking and motivation (Bardgett et al, 2009; Halbout et al., 2019; Wise, 2008). Dopamine's ability to activate reward circuitry has been the driving factor behind drug addiction and eating disorders (Baik, 2013). Understanding how reward circuitry works is an intriguing avenue to travel down. Drugs that increase dopamine, like cocaine and amphetamines are highly addictive through slightly different mechanisms. Cocaine use blocks reuptake causing more molecules to remain in the synapse while amphetamine use produces release via reverse transport sending molecules into the synapse (Egleton & Abbruscato, 2014; Sulzer et al., 2016). Euphoria from increased dopamine levels increase the likelihood of re-engaging in those behaviors (Volkow et al., 2006). There are slightly different interpretations on how dopamine affects reward seeking behaviors. Schultz et al.

(1997) proposes that the dopamine release during reward seeking may not necessarily be due to the reward itself but instead is the result of an error in the anticipated reward. While other research claims that the expectation of drug taking and conditioned cues are enough to prime an individual to activate that system causing reward seeking or drug craving behaviors (Perry et al., 2014). Those abusing drugs experience more pleasurable outcomes when they anticipate taking the drug over unknowingly being given it (Volkow, 2004). Volkow (2004) demonstrates this theory by using cocaine abusing participants and giving them methylphenidate to calculate their brain glucose metabolization with and without the expectational component of receiving the drug. Methylphenidate is the generic form of Ritalin, an ADHD medication that works to increase dopamine by blocking reuptake working in similar ways as cocaine (Kodama et al., 2017; Sulzer et al., 2016). Methylphenidate shows an overall increase in glucose metabolization regardless of expectation when comparing it to placebo (Volkow, 2004). Comparing expectation levels between methylphenidate groups demonstrates a 50 % increase of brain glucose usage in the group expecting the drug (Volkow, 2004). Volkow (2004) demonstrates the increased capabilities that expectation can have on the overall experience of drug taking. Expectation can produce profound alterations that change dopamine's effect in a context dependent manner. In reward seeking, the errors in reward exception produce an effect (Schultz et al., 1997). In drug taking, expecting a drug's effects can magnify the experience (Volkow, 2004). On the other hand, it is important to understand how the expectation of something difficult can alter the likelihood of engaging in that behavior (Mazzoni et al., 2007). If we expect something to be difficult,

we may be less likely to engage in that behavior because of its perceived cost (Mazzoni et al., 2007). Especially when dopamine levels are low like in the case of PD. Expectation affects motivation during the cost/benefits analysis of whether to perform a behavior (Mazzoni et al., 2007).

Dopamine function: Cognition and psychological disorders.

Varying amounts of dopamine generate profoundly different effects. While lower dopamine levels lead to movement impairments (Albanese & Lalli, 2010), excessive amounts of dopamine trigger psychosis and are linked to schizophrenia (Kesby et al., 2018). The dopamine hypothesis of schizophrenia has been supported by the alleviation of positive symptoms with Atypical antipsychotics that target dopamine 2 (D2) receptors and with the phenomenon of drug induced psychosis (Leo & Del Regno, 2000). Medications that increase dopamine, like PD medications or amphetamines can cause drug induced psychosis (Howes & Kapur, 2009; Kesby et al., 2018). Amphetamine use can also cause hyperactivity (Lakhan & Kirchgessner, 2012). These adverse side effects are the result of an overabundance of dopamine. Conversely, Adderall, another popular ADHD medication made of amphetamine salts is given to treat hyperactivity in patients that are believed to have decreased dopaminergic function (Fitzgerald & Bronstein, 2013; Lakhan & Kirchgessner, 2012). Further supporting the sophisticated role dopamine levels play in the brain.

Dopamine function: Motor control.

Movement is an essential part of life. Our brains send signals that regulate movements. Movement can be categorized into two distinct categories, voluntary and

involuntary (Ugawa, 2020). The pyramidal and extrapyramidal tracts are used to distinguish between voluntary and involuntary movements. The pyramidal tract influences voluntary movements and extrapyramidal tract influences involuntary movements (Drobný & Kurča, 2000; Sanders et al., 2012). The term 'pyramidal tract' describes the neural connection from the motor cortex in the frontal lobe that descends through the 'pyramids' of the brainstem to spinal cord. Pyramidal tract axons synapse onto spinal cord neurons that in turn project out of the central nervous system to skeletal muscles throughout the body (Sanders et al., 2012). The extrapyramidal tract in contrast is a different descending motor pathway from the brain to the spinal cord that does not project through the 'pyramids' of the brainstem (Yeo et al., 2014). The extrapyramidal tract includes synaptic pathways through the basal ganglia, and dopamine deficiency in PD may disrupt the extrapyramidal motor system to cause symptoms such as dystonia, dyskinesia, and akinesia (Blair & Dauner, 1992; Sanders et al., 2012). This is supported by the extrapyramidal symptoms experienced by individuals with schizophrenia taking antipsychotic drugs that reduce dopamine levels (Blair & Dauner, 1992). These tracts are important when discussing the differences between voluntary and involuntary movements. Voluntary movements are triggered with the intention of performing a task or in response to a stimulus (Shibasaki, 2011). Voluntary movements originate in the centra nervous system which sends signals to the peripheral nervous system triggering movement (Ugawa, 2020). That information flows from the motor cortex to the spinal cord via motor neurons traveling down descending tracts to convey those messages (Ugawa, 2020). Involuntary movements can represent various actions from basic bodily

functions performed automatically to maintain life, to reflexes, or unintended involuntary movements resulting from dysfunctional systems caused by disease (Ugawa, 2020). Involuntary movements like tremors, dystonia, and chorea, which are rapid irregular twitch-like body movements that are characteristic symptoms of movement diseases like PD and Huntington's disease (Mink, 2003; Ugawa, 2020).

Attempting to understand the mechanism behind motor control has led to the creation of many proposed explanation for the modulation of basal ganglia dopaminergic neurons including modulating motor learning, allowing for motor-error correction feedback during movement, providing the capabilities to retain and utilize motor abilities, and controlling an antagonistic system between the initiation and elimination of movements (Turner & Desmurget, 2010). The 'direct and indirect pathways model' purposes opposing dopaminergic pathways that control movement initiation and inhibition (Calabresi et al., 2014; Isomura et al., 2013). This pathway model incorporates striatal medium spiny neurons (MSNs) that help facilitate its proposed actions (Cui et al., 2013). The direct pathway is said to initiate movement by allowing the thalamus to excite the cerebral cortex (Hikida et al., 2012). The activation of direct pathway is said to causes the cerebral cortex to trigger GABA release activating the striatum that causes glutamate release inhibiting the inhibitory actions of the substantial nigra pars reticulata and internal globus pallidus on the thalamus (Calabresi et al., 2014). Since they are inhibited by the striatum, they are unable to inhibit the thalamus (Calabresi et al., 2014). Meaning the thalamus is free to activate the cerebral cortex and initiate movement (Calabresi et al., 2014). The direct pathway sends projections via the striatonigral pathway to dopamine

receptor 1(DR1) neurons (Gerfen et al., 1990; Hikida et al., 2012). Whereas the indirect pathway is said to extinguish movement by inhibiting the thalamus making it unable to excite the cerebral cortex (Hikida et al., 2012). Occurring through striatal activation that inhibits the external globus pallidus making it unable to affect the subthalamic nucleus (Calabresi et al., 2014). This allows the glutamatergic subthalamic nucleus to excite the substantial nigra pars reticulata which then inhibits the action of thalamus meaning no cerebral cortex excitation (Calabresi et al., 2014). The indirect pathway sends projections via the striatopallidal pathway to dopamine receptor 2 (DR2) neurons (Gerfen et al., 1990; Hikida et al., 2012). This hypothesis has recently come under scrutiny. Various sources have found opposing results that challenge the proposed nature of this pathway model (Cui et al., 2013; Nambu, 2009). Cui et al. (2013) demonstrates concurrent activity in both pathways by utilizing GFP to record Ca+ activity using *in vivo* fiber-optics in mice. The hypothesis also fails to incorporate an explanation for any projections to D3-5 receptors apparent by the outlined projections to solely D1 and D2 receptors (Gerfen et al., 1990). The umbrella terms for D1-like and D2-like receptors do not apply in instances as it is not stated that the D3-5 receptors are activated by the direct/indirect pathways model. Limitations with this hypothesis, suggest that it may not be the most optimal method for examining dopamine's role regarding movement. There is a need for a more precise understanding of how dopamine modulates movement and in the case of PD, motor motivation. This may be possible through identifying specific neuron clusters by using the contemporary hypothesis of movement vigor.

Dopamine function: motivation and movement vigor.

The movement vigor hypothesis proposes the modulation of performance aspects of movement like speed, force, or persistence by dopamine neurons (Dudman & Krakauer, 2016). Thus, the vigor of a specific movement would be influenced by the modulation of dopamine neurons and be dependent on their activity (Panigrahi et al, 2015). Greater activation of dopamine neurons would correlate with more vigorous movements whereas less dopamine activation would trigger less vigorous movements (Panigrahi et al., 2015). Which may be comparable to the type of movement mechanics experienced by PD patients who have decreased dopamine levels. Dudman and Krakauer (2016) proposes a relationship between movement vigor and the implicit form of motivation, a key point in this paper. More specifically, Dudman and Krakauer (2016) identifies a conserved vertebrate feature of the basal ganglia that implicit motivation influences the vigor of movement. Supporting the validity of animal models to test the movement vigor hypothesis across vertebrate species. Movement vigor is the product of motivational components driving behavior (Turner & Desmurget, 2010). From this point on, movement vigor will be used mostly when discussing animal subjects and motivation will be used primary when discussing humans. With a few key exceptions made for literature that uses one term primarily over the other throughout their work. Panigrahi et al. (2015) demonstrates the connection between decreased motivation associated with PD and movement vigor by identifying dorsal striatal neurons that they believe to be vigor related neuron by using a mouse model with decreased dopaminergic function. Panigrahi et al. (2015) supports this by suppressing striatal neurons resulting in decreased

movement vigor. Turner and Desmurget (2010) found that the disruption of basal ganglia function affects movement vigor/motivation altering the execution of movement. Alterations in movement execution can manifest characteristic features of Parkinsonism like bradykinesia and akinesia (Panigrahi et al., 2015; Schilder et al., 2017). Both are forms of motor impairments that can be alleviated using a pharmacological dopaminergic treatment, i.e., L-DOPA (Panigrahi et al., 2015). Demonstrating a link between dopaminergic function, movement vigor/motivation, and PD. Testing this hypothesis could result in a better understanding of dopamine's role in movement modulation and have translational implications regarding the mechanism behind bradykinesia.

One term used to describe this idea is 'PD Apathy', referring to a decrease in enthusiasm or motivation to engage in movement (Mele et al., 2019). Apathy typically occurs in between 17-70% of PD patients (Mele et al., 2019). This paper focuses on dopamine's role in movement vigor, which should be understood as one aspect of dopamine's broader function in motivating behavior. Decreased movement and motivation are based characteristic of lowered amounts of dopamine. The relationship between movement and motivation is therefore of great interest but has been difficult to elucidate in research because the two processes may have overlapping effects on behavior. This section reviews several studies on this topic as background for understanding the proposed experimental approach for isolating the motivation (vigor) component of movement.

Motivation is a multifaceted concept but in this context of movement control it can be understood as non-conscious (implicit) representation of the degree of effort put

into an action. Bradykinesia in PD can be interpreted as a decrease in motivation in this sense, with slower movements indicating lower levels of effort.

This paper focuses on the neurological causes of bradykinesias based on the intriguing theory that there is an innate shift in the motivation to perform movements, not in the ability to perform movements (Baraduc et al., 2013; Mazzoni et al., 2007; Shiner et al., 2012). Mazzoni et al. (2007) uses the term "motor motivation" to explain striatal dopamine projections that influence the motivation to perform movements. This motivation is implicit, happening unconsciously, and should not be confused with the consciously driven explicit form (Mazzoni et al., 2007; Slabbinck et al., 2020). PD patients are not purposely choosing to move slower instead some internal mechanism is driving that behavior. PD may cause a shift in the innate cost/benefit analysis mechanism resulting in decreased motivation (movement vigor) while the ability to accurately perform movements remains intact (Mazzoni et al., 2007). Deceased motivation minimizes vigor or the likelihood of engaging in that behavior. Mazzoni et al. (2007), proposes that movement vigor is a dopamine-dependent attribute that influences the speed of movement, for example, in the task of reaching to pick up an object. Lower motivation (vigor) in PD patients is thought to be due to a change in the perceived energetic cost of performing a movement. This dopamine vigor hypothesis in an alternative to another hypothesis for slower movement (bradykinesia) in PD, the speed-accuracy trade off hypothesis, which suggests that patients slow their movements to maintain accuracy (Mazzoni et al., 2007). Mazzoni et al. (2007) demonstrates a comparable speed-accuracy tradeoff between PD patients and controls that supports the

idea that PD patients still possess the ability to generate swift and accurate movements, but that they are not as intrinsically motivated to do so as are healthy controls. Mazzoni et al. (2007) does this by developing an apparatus intended to study implicit motivation (movement vigor) by recording the speed and movement time of an arm reaching movement between PD patients and controls at various target levels expected to produce varying levels of difficulty. A key component of this task is that participants are given an expectation that their next movement will be either more or less effortful. The apparatus used is multi-level desk like structure with an LCD screen on the top level where the target appears (Mazzoni et al., 2007). The participant's dominant arm is no longer visible to them and the cursor on the LCD screen is meant to be a representation of their arm (Mazzoni et al., 2007). Mazzoni et al. (2007) instructs participants to move their arm when ready towards a bullseye target to record their performance at different difficulty targets. Difficulty was varied using different distance targets that appeared on the LCD screen before each trial. The further targets were believed to be me more difficult because they require more effortful movements (Mazzoni et al., 2007). This method allows Mazzoni et al. (2007) to demonstrate that PD patients are still able to accurately perform fast movements while showing that there may be a dysfunction in the appraisal of the cost/benefit of performing "harder" movements. Building off the work of Mazzoni et al. (2007), Shiner et al. (2012) incorporates the theory of motor motivation into their investigation of bradykinesia. Shiner et al. (2012) develops a reward versus punishment paradigm to test the movement time of PD patients to determine if a motivation shift can be found. PD patients perform faster on punishment avoidance tasks compared with

reward seeking tasks (Shiner et al., 2012), showing that PD patients can move fast when sufficiently motivated, in this case to avoid punishment (electric shocks) (Shiner et al., 2012).

Zebrafish as a model for identifying dopaminergic motivation circuits.

Understanding the specific neuron clusters involved in this motivational shift is hard to do in human subjects, not only for ethical reasons, but because of the complexity of the human brain. In instances like this, utilizing the zebrafish model can be highly beneficial. Zebrafish (Danio rerio) are vertebrates and thus share similar brain structures to other vertebrates like humans (Flinn et al., 2009). Zebrafish provide a promising opportunity for uncovering the mechanisms of neural circuitry. Zebrafish larvae have transparent bodies and brains making them easy to image and study (Higashijima, 2008). Zebrafish frequently produce large quantities of offspring that can be cared for with relative ease given their size (Flinn et al., 2008). Zebrafish demonstrate measurable behavioral responses to stimuli often appearing as changes to their fundamental swimming behaviors (Bishop et al., 2016; Feng et al., 2014; McKinley et al., 2005; Stednitz et al., 2015). Zebrafish have been established as a viable animal model in PD and neurodegenerative disease research. Zebrafish anatomic research and the development of atlas style resources demonstrates many homologous brain structures between zebrafish and humans (Mueller & Wullimann, 2016). Mueller and Wullimann (2016) describes the existences of a shared neural schematic amongst developing vertebrates. Zebrafish brain anatomy demonstrates homologous brain systems and regions that are believed to be dopaminergic controlling movement, motivation, and

reinforcement being present across vertebrates (Flinn et al., 2008; Mueller & Wullimann, 2016). Zebrafish dopaminergic brain system is shown in Figure 3. Majority of the zebrafish dopaminergic brain system is in diencephalon clusters (DC1- DC7) with projections to the telencephalon (Tay et al., 2011). In the ventral diencephalon (vDc), the posterior tuberculum is believed to be the closest zebrafish homolog to the substantia nigra in humans (Flinn et al., 2008; Nellore & P., 2015; Stednitz et al., 2015; Xi et al., 2011). The region is highly saturated with dopaminergic neurons and is a key area of interest in PD research using zebrafish. Ascending projections would be carried to the subpallium of the ventral telencephalon, a proposed basal ganglia homolog of the striatum (Mueller & Wullimann, 2016). There is some limitation to using zebrafish as a model organism for humans. Not all regions of zebrafish brains are directly homologous to human brains as previously mentioned (Stednitz et al., 2015). Zebrafish do possess most of the subcortical motor structure typical of mammals is a particularly important for motor control, including determining movement intentions (e.g., moving toward or away from a stimulus) and in being the origin of the corticospinal motor tract discussed previously (Knierim, 2020). The distribution of dopaminergic neurons varies slightly between zebrafish and human brain regions. The greatest differences between zebrafish and human brain anatomy are in the forebrain (Mueller & Wullimann, 2016). Zebrafish do not possess midbrain dopaminergic neurons like humans instead homologous region are located within the forebrain (Tay et al., 2011). Aside from the anatomical differences, higher order processing carried out by mammalian forebrain regions have a higher degree of complexity compared to zebrafish (Mueller & Wullimann, 2016).

Zebrafish still possess sufficient similarities to be useful animal models for the type of research on subcortical motor systems being proposed in this paper. Though some limitations exist, zebrafish have been successfully used in PD research with various methods (Feng et al., 2014; Matsui & Sugie, 2017; McKinley et al., 2005; Stednitz et al., 2015; Xi et al., 2011). An extensive search of the current literature demonstrates that researchers have studied PD in zebrafish by utilizing neurotoxins and pesticides to elicit disease-like symptoms and transgenics to observe brain structures and physiology. The neurotoxins 6-hydroxydopamine **(**6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are two commonly used and widely accepted chemicals that induce PD-like symptoms in the zebrafish model. 6-OHDA possesses the ability to destroy dopaminergic and noradrenergic neurons by utilizing neurotransmitter reuptake to enter and disable those neurons (Matsui & Sugie, 2017; Feng et al., 2014). In PD pathology, it is well established that there is a decrease of around 70% of the total concentration of dopaminergic neurons in the substantia nigra. Various studies on zebrafish have reliably demonstrated MPTP's ability to decrease the concentration of dopaminergic neurons to desired levels for modeling PD (McKinley et al., 2005; Xi et al., 2011). MPTP metabolizes into 1-methyl-4-phenylpyridinium (MPP+) which damages dopamine neurons by latching on to the dopamine transporter protein (Matsui et al., 2009). Similarly, rotenone, a farming pesticide, has recently been established as another viable method which can induce both motor and non-motor PD-like symptoms in zebrafish (Wang et al., 2017). Rotenone successfully disrupts motor function by decreasing the duration and distance zebrafish travel at fast speeds (Wang et al., 2017).

Using a light/dark preference test anxiety and depression-like behaviors in zebrafish that are identifiable by the time spent in the light box and an unwillingness to travel into the dark one (Wang et al., 2017). Impairing dopamergic function in zebrafish causes characteristic behavioral alterations that are comparable to those in humans.

Transgenic zebrafish, in which genes are introduced to express fluorescent markers or effector proteins in specific neuron types, have become a widely utilize tools in behavior neuroscience research (Burket et al., 2008; Higashijima, 2008). Transgenics is the process of transferring a gene from one organism into another (Higashijima, 2008). Higashijima (2008) identifies a current method of creating transgenic zebrafish by inserting a DNA construct into the genome of a zebrafish embryo at the single cell point in development. Many transgenic zebrafish lines have been created expressing fluorescent proteins in a variety of manners from encoding transporter proteins, enzymes, ions, and other molecules of interest (Gao et al., 2005; Muto & Kawakami, 2013; Stednitz et al., 2015; Xi et al., 2011). Using transgenics that express fluorescent proteins, like green fluorescent proteins (GFP) improves the capabilities of differentiating neurons of interest in the brain (Higashijima, 2008). Some transgenic zebrafish, like the GCaMP line possess genetically encoded calcium indicators (GECIs) that express GFP with the ability to detect calcium (Ca2+) ions in a voltage dependent manner (Muto & Kawakami, 2013). All the methods mentioned support the capabilities of performing PD research using zebrafish and help demonstrate the versatility of zebrafish as a behavioral neuroscience model organism.

Statement of the Problem

There is a need for a better understanding of dopamine's modulation of motor function. The decreased motor motivation that PD patients experience demonstrates the presence of an underlying mechanism that needs further investigation. The ability to accurately perform movements yet the inability to engage in those movements is a puzzling phenomenon. The movement vigor hypothesis, which suggests that some dopamine neurons in the basal ganglia specifically control vigor components of movement such as speed, persistence, and force, could account for this pattern of movement deficits as well as theoretical models of dopamine's role in motor control in general. The current study would use experimental methods in zebrafish to identify any neurons with specific vigor-related functions. The zebrafish model offers a unique and powerful suite of methods for studying behavioral functions of neuron, and as vertebrate, direct homologies can be inferred between neurons in zebrafish and human brain, allowing these studies in zebrafish to form the basis of hypotheses about the function of specific homologous neurons in human. Identifying vigor-control neurons in this way could help treating certain human motor disorders.

Given the current Coronavirus (COVID-19) pandemic and the institution of social distancing, limitation have arisen that may impede my ability to conduct the proposed research in this paper. Due to the current uncertainty that exists this paper will outline a study that may or may not take place. The current pandemic has brought with it restrictions that prevent performing experiments in a laboratory setting, which is crucial in obtaining the data needed for this proposed study. These uncontrollable circumstances

may affect data collection and the ability to complete this study in a timely manner. Thus, this paper is written in the hypothetical, proposing various outcomes based off the current literature and the broader implications they possess. Existing data from Dr. Gahtan's laboratory will also be analyzed. Both behavioral and neuroimaging data will be used for the analysis.

The proposed study

The goal of this proposed experiment is to use some of the unique advantages of zebrafish for neural circuit mapping to identify the specific dopamine neurons subgroup that encodes the vigor/motivational component of movement. Since previous research suggests that dopamine deficiency disrupts movement vigor most when there is an expectation that a forthcoming movement will be difficult, I propose using a behavioral test in zebrafish that attempts to manipulate the fish's expectation of how difficult (specifically, how long in duration) a forthcoming movement will be. This behavior, the dark flash evoked diving response, reliably persists as long as the dark flash stimulus (sudden light dimming), so the longer the stimulus the longer and more difficult (effortful) the behavior. After repeated trials of long or short dark flash stimuli, we hypothesize that larvae will form an expectation that a subsequent trial, when dopamine neurons were tested, will require the same degree of effort. By varying this component of expected effort, I hope to isolate neurons whose activity is correlated with, and is necessary for, controlling the motivation to meet movement demands.

There are four main phases to the proposed experiment: (1) behavioral tests using the dark flash paradigm to determine whether zebrafish larvae form expectations about

forthcoming movement demands. Evidence of the expectation will come from comparing dive performance between zebrafish trained with a repeated short versus long dive challenge. If test dives under equivalent conditions are more persistent or faster after training long dive training, that will show that a history of trials of a certain difficulty led to an expectation that subsequent trials will be similarly difficult; (2) neural recordings during dark flash evoked dives will be used to identify neurons whose activity is correlated with changes in movement vigor; (3) lesion studies where candidate motivation-encoding neurons identified in phase 2 are selectively inactivated followed by behavioral tests to determine whether those neurons were necessary for normal modulation of movement vigor; (4) anatomical and molecular analysis of candidate vigor neurons to determine homologies with human basal ganglia neurons groups. This will be done by matching neuron groups revealed in phase 2 and 3 with equivalent groups in the Zebrafish Brain Browser atlas (http://vis.arc.vt.edu/projects/zbb/), which provides the anatomical identity and molecular phenotypes for most neurons throughout the zebrafish larva brain (Tabor, et al., 2019). It is important to note that the logic for conducting phase 2, 3, and 4, depends on support of the hypotheses of the previous phases. Therefore, if zebrafish larvae show no evidence of forming expectancies of movement difficulty, subsequent phases may not be warranted, and the information from this study may be limited to the question of expectancy formation in zebrafish. A breakdown of each phase is depicted in Figure 1.

Research Hypotheses and Rationale

Hypothesis 1a. Zebrafish larvae will demonstrate expectations about movement vigor demands after training.

Hypothesis 1b. Six day old zebrafish larvae trained with 50 repeated, 30-sec dark flash evoked dive training trials (high vigor demand), will show more dive persistence, speed, and distance (3 related measures of movement vigor), than an equivalent group of larvae trained with 50 repeated 5-sec dark flash training trials (low vigor demand), when tested in a single, post-training 60-sec dark flash challenge trial.

Rationale for Hypothesis 1. Zebrafish larvae are known to adapt the intensity of swimming motor output based on visual feedback (Ahrens et al., 2012), demonstrating a similar type of sensorimotor adaption anticipated from the different training dive durations in the prosed experiment. In this previous research, Ahrens et al. (2012) also interpret changes in zebrafish motor behavior as being based on expectation (for example, they wrote “When visual feedback following a motor command does not meet expectation, animals can learn to adapt the strength of subsequent motor commands.”), therefore the current hypothesis that zebrafish will form motor output expectancies is based on a solid precedent.

Hypothesis 2. Dopamine neurons in the brain will show different patterns of activity during dive behavior, as measured by neuronal calcium imaging, following training for low versus high vigor demand.

Rational for hypothesis 2. Transgenic zebrafish expressing the CaMPARI2 photoconverting calcium indicator will be used in these experiments. This fluorescent

protein is expressed in all neurons and permanently marks all active neurons at a specific moment in time (when the brain is exposed to UV light) through a green-to-red fluorescent photoconversion is permanent, larvae can be imaged later, after the behavior trail, to determine which neurons were active during the previous dive behavior. By comparing activate neurons in larvae with low versus high vigor demand expectations (based on prior training, which would be the only differences between those groups of larvae), this experiment can isolate neurons that are differently activate based on vigor demand expectancy. While I expect the differential neural activity to be localize to dopamine neurons cluster based on previous research implicating dopamine in movement vigor, this method will image activity throughout the brain, so vigor-related neurons may be found elsewhere. Therefore, only the limited hypothesis is being made that there will be a difference in overall neural activity based on vigor expectancies. CaMAPRI2 photoconversion in zebrafish is illustrated in Figure 2.

Hypothesis 3. Laser ablating neurons with vigor-related activity will disrupt movement performance, specifically, movement persistence, speed, and distance, when vigor demand is high but not when vigor demand is low.

Rationale for hypothesis 3. This hypothesis is based on parallel findings in human PD patients showing deficiency when the expectation of the movement would be high and comparable performance to controls when the expectation of the movement would be easy (Mazzoni et al., 2007). Larvae trained with short (5-sec) dives should expect the test dive to also have a low vigor demand, while larvae trained with 30-sec dives should expect a higher vigor demand. The motivation deficit in human PD patients

has been interpreted as a kind of apathy or a reluctance to engage in effortful action, therefore, I expect the behavioral deficit in zebrafish to be limited to conditions where the forthcoming action is anticipated to be more effortful or vigor-demanding. This change is expected to manifest itself as decreased dive persistence and distance.

Hypothesis 4. Vigor-related neurons identified through neural activity and lesion studies will have anatomical and gene expression properties that allow homology mapping to specific neuron types in the human brain.

Rationale for hypothesis 4. I expect a limited, sparse, and anatomically identifiable subset of neurons to show vigor-related activity in the experiment described for hypotheses 2 and 3. The online Zebrafish Brain Browser atlas (http://vis.arc.vt.edu/projects/zbb/), and the Atlas of early zebrafish brain development (Mueller & Wullimann, 2016), as well as numerous published articles, provide information about the gene expression profiles of most neuron groups in the larval zebrafish brain. The vigor related neurons this study identifies can be matched by anatomy to neurons listed in these atlases, and then connected to gene expression information from the atlas such as whether a neuron expresses the dopamine synthesizing enzyme, tyrosine hydroxylase, as is therefore a dopaminergic neuron. This combination of anatomical and molecular features should allow our vigor related neurons to be linked to homologous neurons in the human brain.

Methods

Hypotheses 1- 4 present an ordered progression of studies so methods are presented in that order.

Behavioral measures of movement vigor expectancy

Hypothesis 1 is that zebrafish larvae will show behavioral evidence of the formation of expectancies of required movement effort based on previous training experience. The operational definition of movement vigor expectancy will be 3 parameters of a visual stimulus-evoked diving response: dive latency, speed, and distance. Two groups will be trained to form a high or low movement vigor expectancy based on the duration of 50 previous, training dive trials. Dark flash stimuli lasting 5-sec or 30-sec will be used to train low or high movement effort expectancy, respectively, in separate groups in a between-subjects design. Each training trial will consist of a 2-minute light-on pre-trial period during which overhead, diffuse white light illumination remains on, followed by a dark flash stimulus during which the light is extinguished and remains off. Fifty training trials will be run with a 2-minute, light-on intertrial interval between dark flash stimuli. Measurements of dive latency, speed, and distance will be made on a single test dive immediately following the final training dive. The test dive will use the longer, 30-sec dark flash stimulus for all subjects, with the hypothesis that the low vigor expectancy group will demonstrate less vigorous dives (longer latency to dive onset, slower dive, and less negative vertical displacement) than the high vigor expectancy group. Each larva will be tested in an individual, 50x10cm round plastic tube containing 50ml of regular fish water. This vertical distance allows sufficient range for a

30-sec dive when larvae start toward the top of the water column, which is expected because larvae tend to swim at the top of the tube during light-on conditions. Multiple tubes will be lined up and video recorded at a rate of 1 frame per second, allowing up to 10 larvae to be simultaneously tested. An example of this procedure from a pilot experiment is shown in Figure 4.

Vertical distance on each video frame will be measured from the recorded test dive using image analysis tools in ImageJ (Schindelin et al., 2012). Dive latency will be measured as the time post-stimulus that the larva begins a downward swim that persists for at least 4 consecutive seconds. Dive speed will be measured as the total negative vertical displacement (in mm) divided by the total number of seconds of dark flash stimulus. Dive distance will be measured as the total number of mm of vertical displacement from the start to end of the dark flash stimulus. Larvae will be trained and tested at 6 days of age. Larvae in low and high expectancy groups will be siblings, tested on the same day, to minimize individual differences unrelated to training that may affect performance in the test trial. Each group will consist of N = 17 larvae for adequate statistical power based on a power analysis (Power = 0.8, $\alpha = .05$).

Measuring neural activity during evoked movements

A common method for imaging neural activity in zebrafish larvae is the use of genetically encoded calcium indicators (GECIs). This method involves a foreign gene (a transgene) that encodes a calcium-sensitive fluorescent protein, being introduced into the zebrafish genome, resulting in neuron-specific expression of a fluorescent protein that responds to neural activity-related calcium increases by briefly becoming brighter. In

most cases zebrafish larvae are restrained in agar gel to stabilize their brains for imaging. Since many behaviors cannot occur in restrained larvae, this method limits the ability to relate neural activity with different behavioral states. The experiments proposed to test Hypothesis 2 would use an extension of GECI technology, called CaMPARI2, that allows neuron activity to be recorded in freely swimming larvae, with the limitation that neural activity can be recorded only within a brief moment in time, approximately several seconds. CaMPARI stands for 'Calcium-Modulated Photoactivatable Ratiometric Integrator'. Briefly, this protein binds calcium so it is sensitive to neural activity in the same way as traditional GECIs, but instead of indicating activity as changes in brightness, calcium induces a permanent green-to-red photoconversion of the protein. However, that photoconversion only occurs in the presence of UV light (~405 nm), which allows only those neurons that are active during the UV light pulse to be marked as red. Then the brain can be imaged later in a restrained larva to determine which neurons were active when the UV pulse was shown.

Using CaMPARI2 zebrafish in the current experiment, neurons that are active during dark flash test dives will be marked by flashing the UV light 5 sec following the dark flash stimulus (when the dive is likely underway), for a period of 1 second, marking all neurons active during the dive. After the test trial larvae will be immediately embedded in agar gel and neurons will be imaged using a confocal microscope in both red and green fluorescent channels. Activity in each brain region will be quantified as the ratio of red (active) to green (non-active) neurons. The key brain activity comparison will be between the groups trained to have low versus high effort expectancy during the test

dive. Brain regions that are more active in the high effort expectancy group will be considered candidate vigor-encoding neurons. Each group will consist of N = 11 larvae for adequate statistical power based on a power analysis (Power = 0.8, $\alpha = .05$).

Simulating CaMPARI2 calcium imaging during dark flash trials

CaMPARI2 photoconversion will be simulated using 30 existing confocal stack images of zebrafish larval brains. It is important to note that this simulation will be generated from data recorded under slightly different conditions than the proposed experiment. Existing images are of data collected on immobilized zebrafish larvae during dark flash trials. Each image will begin as confocal stack of 60 frames, representing 1 frame per second. Images will be randomized by Dr. Gahtan and assigned to individual folders with unrelated names before any modifications are made. The CaMPARI2 simulation will be performed using ImageJ. Metadata for each image will indicate the event frame where elevated levels of Ca2+ occur. The event frame will serve as the point of reference for performing the transformative procedures. Two new images will be created from the original confocal stack image using the Z-project function. The new images will be averages of 10 frames +/- 2, before (Image 1) and after (Image 2) the event frame. This decision was made in accordance with the temporal nature of Ca2+ unbinding kinetics. Image 1 will act as a baseline for its respective trial. The image calculator function will be used to generate the result of subtracting the two averaged images. The result of the subtraction image and Image 1, the baseline image will be used to create the CaMPARI2 visual representation. The merge channel function will be used to color coordinate the appropriate images by assigning them to a special color channel

generating the CaMPARI2 simulation. The use of the red and green color channels was chosen to mirror the photoconversion of CaMPARI2. This function will produce two images on top of one another. Next, the overlay function and flatten tool will be used to condense the two images into one composite RGB image that will be ready for analysis.

CaMPARI2 simulation analysis

The purpose of using CaMPARI2 is to provide a quantifiable ratio of red to green photoconversion that can identify active neurons during an intended behavior. Simulating CaMPARI2 using the red and green color channels allowed for similar methods of data analysis to be performed. A region of interest (ROI) analysis was performed on data collected and modified using ImageJ. As previously mentioned, dopaminergic neurons are believed to control movement vigor. In zebrafish, the dopaminergic system is located mostly in the diencephalic clusters (DC1-7) which was included in the ROI. Anatomical data from the online Zebrafish Brain Browser atlas (http://vis.arc.vt.edu/projects/zbb/) was used to identify and create the ROIs according to documented zebrafish brain anatomy. ROIs were individually drawn for each zebrafish in the CaMPARI2 simulation. Various zebrafish brain images were used for this simulation which were taken under different magnification. This led to a variation in pixel size, image size, and standard deviations between ROIs across images. All this information was documented and was a limitation of this work. The area of each ROI was calculated in ImageJ. Independent samples T-tests were performed to compare the average ratio of red to green pigmentation between larvae assigned to the low and high effort conditions. Images were taken from existing data in Dr. Gahtan's laboratory on BPA treated and control larvae.

Larvae were treated by Dr. Gahtan and assigned to randomized folders to ensure random assignment was not compromised. The CaMPARI2 simulation included a color adjustment that was performed in ImageJ and was intended to act as a visual representation for the process of CaMPARI2 photoconversion.

Testing the formation of expectancies about elicited locomotor responses in zebrafish larvae using existing data

In December, 2019, I was part of a research team in Dr. Gahtan's laboratory that began pilot studies of associative learning in zebrafish larva under an approved IACUC protocol titled 'Development of associative learning in zebrafish' (HSU IACUC #2019P35). COVID-19 campus closure stopped my work on this project within about 2 months, but I performed one experiment. I analyzed the data instead for this study in a manner not originally planned. This enabled me to test one hypothesis related to the formation of motor effort expectancies in zebrafish larvae. This experiment was a pilot run of procedures that I conducted as part of my work toward a Pavlovian conditioning study in which a tone conditioned stimulus would be paired repeatedly (in consecutive trials) with a dark flash unconditioned stimulus. The dark flash, as described previously in this thesis, elicits a dive as an unconditioned response. The goal was to test whether the tone would come to elicit dives as a conditioned response, but the experiment I actually recorded while developing procedures for this study consisted only of repeated dark flash dive trials without a tone. Specifically, 4, 6-day old zebrafish larvae were recorded simultaneously in adjacent vertical half-pipes (50cm x 10cm white PVC tubes split in half with a flat glass window glued on for imaging, containing ~20ml of fish water)

across 20 dark flash trials, each lasting 10sec with a 5min intertrial interval (Figure 4.). The tubes were facing an infra-red (IR) sensitive machine vision camera (Pixelink PL-A 641) equipped with a visible light blocking filter. The setup was located inside a sound- and light- attenuating cabinet that also contained a heater set to 28deg C (to maintain appropriate fish water temperature), an IR lamp for video imaging, and a white-light LED array above the tubes used for the dark flash stimulus. The camera and stimulus light were controlled by a custom computer program written in DacFactory (Azeotech) and were connected to the computer through a LabJack U3 (Digikey) input-output interface.

My proposed analysis was based on the reasoning that if expectancies about the motor demands of the dive behavior form across these 20 trials, then kinematic measures related to dive effort should change across the trials. I specifically tested for changes in dive latency (time post-stimulus of dive onset), and average slope referred to as dive displacement (rise/run averaged across the dive duration) as a function of trial number, using a one-way repeated measures ANOVA with trial number as the within-subjects factor. Trials were blocked together in 5, 4-trial blocks. I operationalized latency as the time point post-stimulus on which at least 3 seconds of uninterrupted negative vertical movement had begun. I operationalized dive duration as a span of at least 3-sec post-stimulus across which there was uninterrupted negative vertical movement. I used a two-tailed test of the effect of trial block on dive displacement because my only hypothesis was that, if expectancies form, larvae would adapt their latency or speed based on their experience in previous trials. I have no a priori reason to believe that latency or speed will necessarily increase or decrease in these conditions.

Diving behavior was quantified in ImageJ to determining the Y axis (vertical) position of each larva on each video frame. This provided a sequence of 35 frames per trial in an image stack. Thresholding was performed in ImageJ to isolate each larva (Figure 5). Images in each video stack were subtracted from the previous image to isolate pixels that had changed in brightness from frame-to-frame since the larvae were the only objects moving. Then, the desired post stimulus frames were extracted for calculation.

Figures

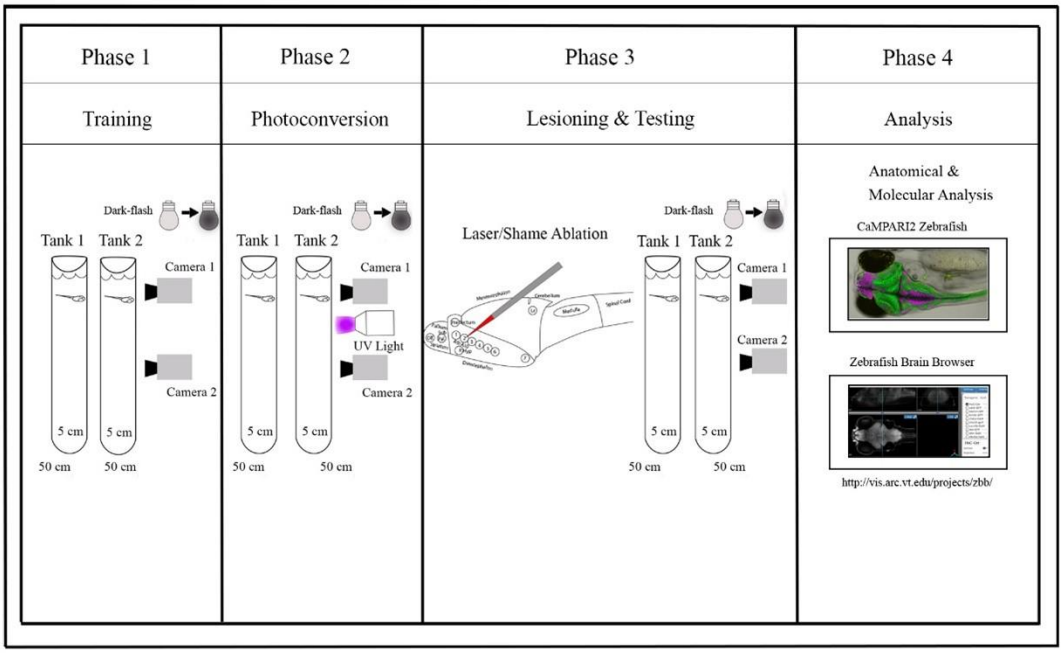

Figure 1. Experimental phases.

Phase 1: Transgenic CaMPARI2 zebrafish larvae will be placed into one of two deep dive conditions for training (easy or hard). Training should condition an expectation of dive difficulty dependent on the condition subjects are trained in. Following training CaMPARI2 photoconversion will be triggered using a UV light (~405 nm).

Neuroimaging techniques will be performed to capture pan-neuronal images to identify potential vigor related neurons. Phase 2: Following identification of candidate vigor related neurons, lesioning will be performed through laser ablation of target neurons on new sets of CaMPARI2 zebrafish larvae. Two distinct laser ablation techniques will be used (laser ablation/shame ablation). The use of a shame laser ablation will act as an inactive tool to maintain the closest design parameters. This will be done to formulate causal evidence on whether these candidate neurons encode movement vigor. Three subgroups will be generated for further examination (lesion/hard, lesioned/easy, shame lesioned/hard). Phase 3: Training of the three subgroups will follow the same training protocol used in phase 1. Data collection will be performed to generate comparable data between groups to determine if candidate vigor related neurons influence various aspects of movement (speed, persistence) across conditions.

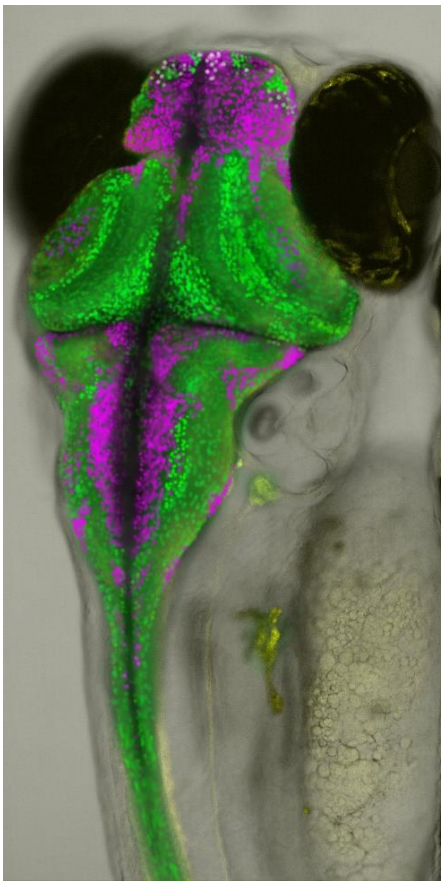

Figure 2. CaMPARI2 photoconversion illustration.

CaMPARI2 Photoconversion Illustration demonstrating pan-neuronal recording capabilities allowing for the identification of specific neurons active during a behavior. CaMPARI2 photoconversion permanently converts active neurons from green-to-red in the presences of a UV light (~405 nm) and elevated levels of intracellular Ca2+. With the uncertainty surrounding laboratory access, an illustration was created to demonstrate the capabilities of this tool. This illustration depicts the pan-neuronal nature of CaMPARI2 and was generated using a reference image of a transgenic CaMPARI2 zebrafish brain taken from Moeyaert et al. (2018). The illustration was created in Adobe Photoshop and is overlain on a zebrafish larva image taken in Dr. Gahtan's laboratory.

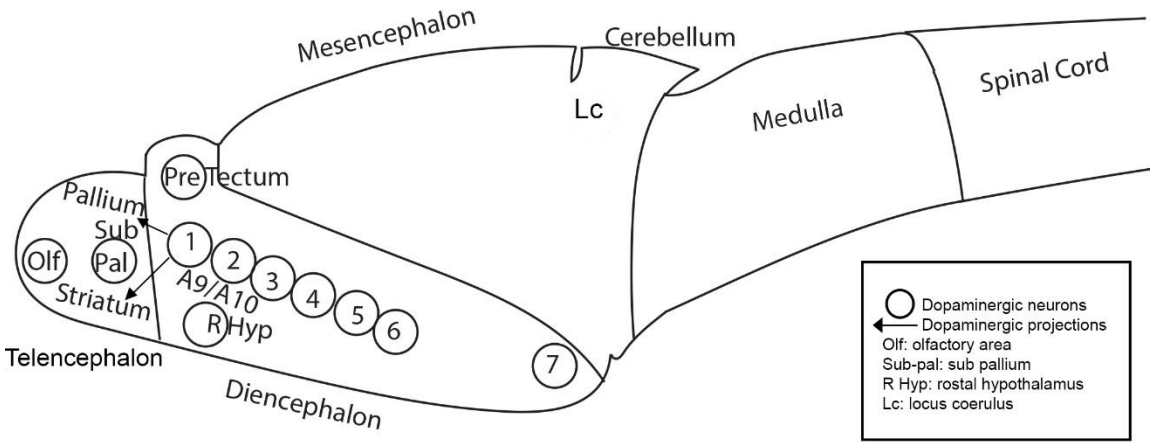

Figure 3. Zebrafish brain anatomy.

Zebrafish anatomy demonstrates transverse neuromeres that are preserved across vertebrate species (Mueller & Wullimann, 2016). Homologous vertebrate brain regions are present in zebrafish. Vertebrate species possess a conserved dopaminergic system shown in the zebrafish brain anatomy figure above. Slight differences exist across vertebrate species, in zebrafish the dopaminergic system is mostly located in the forebrain compared to its mostly midbrain location in humans. The appearance of the dopaminergic system is highlighted within the diencephalon clusters that has projections to the telencephalon.

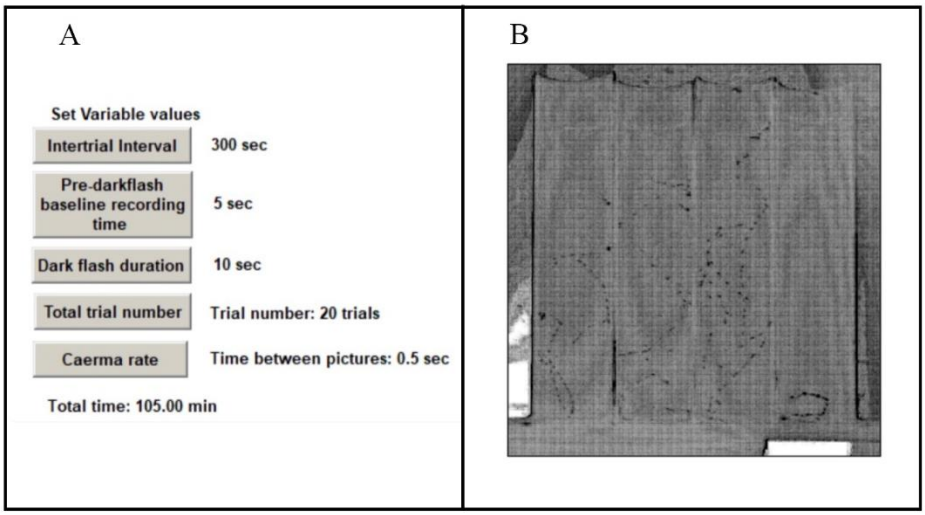

Figure 4. Experiment parameters for repeated dark flash dive trials.

Note: (A) Screenshot of the DacFactory (Azeotech) program interface used for the original dark flash test trials run on 2/14/2020. Parameters are shown for the experiment and were automatically repeated by the program until the completion of the final trial. (B) Image of the recording setup showing the 4 tubes and swimming paths of larvae across 100 video frames. A total of 20 trials were run on 4 zebrafish larvae. Vertical dive tubes were placed in a sound- and light- attenuating cabinet for dive recording. An LED array was set up in the cabinet and automatically activated by the program to elicit the diving behavior.

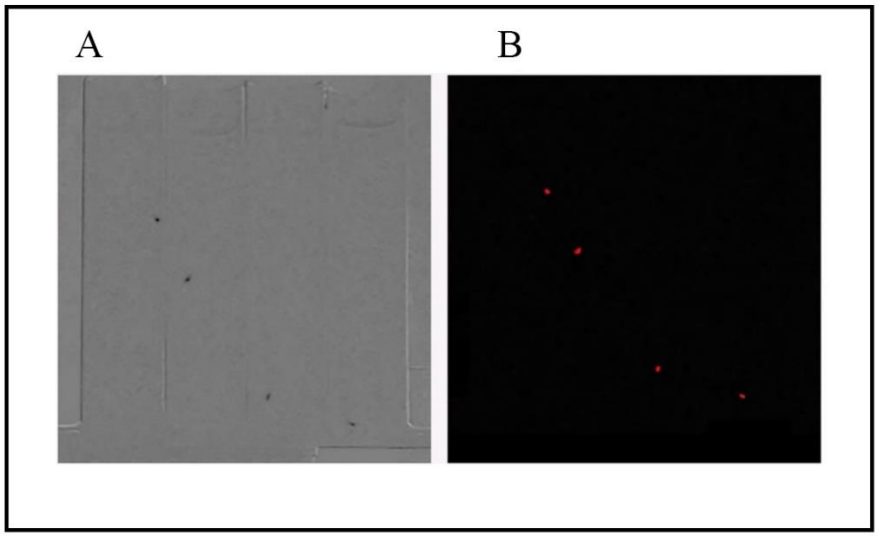

Figure 5. ImageJ manipulation, subtraction and thresholding.

Images of previously described dark flash trials run on 2/14/2020. Both images represent the same diving sequence of 4 zebrafish larvae in 50cm x 5cm vertical deep dive tanks. Larvae were recorded over the span of 20 trials using video recording. Both images present an example of the ImageJ manipulation used to collect quantifiable data for comparisons of variables during experimentation. The image on the left (A) is a subtracted version of the original video frame improving the accuracy of data collection. The image on the right (B) is set to a threshold to further minimize the probability of interpreting incorrect data caused by noise. The red pigments represent 1 of the 4 larvae in the experiment.

Results

On the CaMPARI2 simulation images, a region of interest (ROI) corresponding to the diencephalic dopamine neurons cluster (Figure 3) was individually drawn in each brain (n = 30). The size of this ROI was relatively consistent across brains (M = 13,372.44 ± 4914.12 pixels), supporting direct comparisons across brains. Independent samples t-tests were performed to compare the average red to green 'photoconversion' ratio in the ROI between larvae in the simulated low and high effort conditions. The average red to green 'photoconversion' was not significantly different between the low (M = .43, SD = .16) and high (M = .46, SD = .13) effort conditions, $t(28)$ = .52, 95% CI [-.0789, .1331], p = .61, d = .19.

The behavioral dive data was used to examine Hypothesis 1 by looking at the displacement of the zebrafish during repeated dark flash trials (n=4). Dive vigor was determined using the Y coordinate data for each fish to calculate the slope for the frames that occurred after the stimulus. A one-way repeated measures ANOVA was performed with trial number as a within-subjects factor which represented the 20 repeated trials. Dive slope for each subject was: Fish 1 (M = .11, SD = .10), Fish 2 (M = .11, SD = .12), Fish 3 (M = .14, SD = .09), and Fish 4 (M = .35, SD = .17). This experiment was performed to determine whether larvae form an expectancy for motor effort. This was done by examining whether dive latency increased following repeated trials, specifically, by finding a main effect of trial number in the repeated measures analysis. Dive latency did not significantly increase with repeated trials, $F(4, 12)$ = .37, p = .83, ges = .059. If an increased latency would have been recorded it would have most likely been in response

to their previous experiences, which would constitute some evidence that zebrafish larvae can form an expectancy of the effort required for a forthcoming dive based on their experience in preceding trials. This would have supported the hypothesis that larvae can form this type of effort expectation in regard to motor function. Since this was not supported by the data the subsequent hypotheses would not require further analysis.

Discussion

It is important to keep in mind that the data analyzed in this paper were collected under slightly different parameters than the proposed study and should be interpreted as such. Differences in dive training, sample size, and neuroimaging may have influenced these results. Dive training was not possible due to the use of previously collected data that did not incorporate prior trial training. The sample size needed to achieve the desired power (Power = 0.8, $\alpha = .05$) for the proposed study was not possible due to the lack of available subjects in the previously collected data. Neuroimaging was not performed on transgenic CaMPARI2 larvae and instead a simulation was used to illustrate the expected photoconversion. These are some of the many differences that exist between the proposed study and the data analyzed in this paper. These differences could have caused disparities between these results and those of the proposed study. Since performing the proposed study was not achievable many potential questions remain. Upon completion of the proposed study, behavioral data would have determined candidate vigor neurons that would have been laser ablated to determine their function. Data analysis would have determined whether those neurons were dopamine neurons and if they did in fact encode movement vigor. If so, further analysis would have been performed to identify the specific dopaminergic neuron clusters that were more active during greater degrees of vigorous activity. Data would have demonstrated whether the expected diencephalic clusters (DC1 - 7) were active or not. This would have illustrated if movement vigor could be mapped on to a specific set of neurons. The proposed study would have also demonstrated the efficacy of utilizing CaMPARI2 photoconversion. This technology is

new and has not been widely utilized in the literature yet. The proposed study would also have incorporated laser ablations of potential candidate neurons that may or may not have been dopaminergic. This would have provided the means to draw causal relationships from the correlational behavioral data that would have been collected. Repeated dive trials would have demonstrated whether zebrafish larvae are able to form the expectation of the energy output needed based on previous trials and how lesioning would have affected their activity.

Attempting to address all these components of the proposed study would have been extremely difficult without actual data to analyze. That is the reason I analyzed and interpreted the results from a previous set of dive trials and performed the CaMPARI2 simulation which helped demonstrate what the results from the proposed study may have looked like, though the conclusions from that data may or may not accurately depict the findings of the proposed study. This interpretation and visual representation are to demonstrate what data could have looked like and elaborate on an outcome that could have occurred in the actual study. With the limited access to the laboratory this method was used to demonstrate the steps that would have been performed to collect, transform, analyze, and interpret the data.

The results of the CaMPARI2 simulation produced an illustration that resembled what actual CaMPARI2 photoconversion looks like in the zebrafish brain (Moeyaert et al., 2018). The ratio of “active” and “non-active” neurons fit within the range of what we would expect in a ROI analysis. Prior to performing statistical analysis, the visual inspection of the data aligned with the expected ratio parameters. The number of red

pigments detected did not surpass the number of green pigments. This result was encouraging because it showed that only a restricted subset of neurons in the brains were activated by the dark flash stimulus. In contrast, if most of the brain had shown activation, that would be surprising and suggest a conceptual or technical error in my simulation approach. Larvae were assigned to low and high effort conditions randomly with no pre-training as in the experiment that was proposed but not able to be conducted, so a lack of simulated group effect in my analysis was expected, and if a statistical group difference had been detected it would not have been necessarily by chance since the group assignment was random (i.e., simulated).

Examining the data from the previously recorded associative learning dive trial demonstrated that zebrafish reliably dive to the bottom of tank when presented with a dark flash stimulus, findings that aligned with previous published work done by Dr. Gahtan's laboratory (Bishop et al., 2016). Even though the data analysis performed in this paper did not support the hypothesis that zebrafish larvae could form the expectation of dive difficulty, it does not rule out the possibility that zebrafish larvae can actually form expectancies. Without actually performing the repeated dive trials we cannot determine for certain whether or not larvae would respond differently than what was previously reported in this paper. A lingering limitation to this work is that larvae were tested under similar dive conditions and were not assigned to either a low or high effort conditions. Thus, the results may not fully represent what that outcome would have been if that had been possible. Future work could re-examine this hypothesis with larvae assigned to low and high effort conditions to evaluate whether or not larvae form an expectation of motor

effort through the diving trials. An increased dive latency would demonstrate that zebrafish larvae were able to interpret the difficulty of the dives by adapting to meet the energy requirements of their surroundings, as suggested by previous studies (Ahrens et al., 2012). This would be supported by the increased dive latency that was not present in earlier trials. Identifying whether larvae can form the expectation of dive difficulty would support their use as a research tool for identifying vigor-related neurons. This would ultimately help determine which neurons encode movement vigor which could be used as targets for improving PD associated motor impairments.

Conclusion

PD is a debilitating neurodegenerative disease that can negatively affect an individual's quality of life. Motor impairments are a key component of PD. In the literature the link between dopamine neurons and motor control has been made. Decreased dopaminergic activity has been attributed as the primary cause for motor impairments. The primary hypothesis regarding dopamine's modulation of movement is the direct/ indirect pathways model that proposes two opposing movement pathways. Recent research has suggested that limitations exist bringing this hypothesis into question. Examining alternative hypotheses could yield novel insight into treating movement related disorders like PD. Mazzoni et al. (2007) identified an intriguing phenomenon that a motivational shift occurs in PD patients causing bradykinesia. Patients move slower when the task is perceived difficult while the ability to move fast remains preserved. This is attributed to a shift in the cost/benefit analysis of performing forthcoming movements. Mazzoni et al. (2007) utilized the movement vigor hypothesis to examine the performance of PD patients and controls across varying levels of expectation difficulties. The movement vigor hypothesis proposes that dopamine neurons control performance aspects of movement. In this case, movement speed in the form of bradykinesia in PD patients. Studying how dopamine neurons control movement vigor could be used to identify whether certain aspects of motor control are mapped on specific neurons. Understanding which neurons are linked to aspects of movements can be used to repair signals and improve function.

The proposed study would have examined dopaminergic activity using the movement vigor hypothesis to identify vigor-related neurons. Since zebrafish are vertebrates, they share many homologous brain regions and systems with humans. The presence of a dopaminergic system makes zebrafish an ideal candidate for examining the movement vigor hypothesis. Zebrafish have also been shown to reliably perform a diving behavior when presented with a dark flash stimulus. The nature of this behavior makes it ideal for identifying vigor-related neurons. Repeated dive trials demonstrated that zebrafish in this sample did not form an expectation for motor effort. Though longer duration dark flashes were not tested. Dive speed and persistence were also traded in for a single measurement of displacement that was derived by calculating the slope of each dive. Calculating speed and persistence may have produced a different depiction of the degree of vigor required during each dive. Labeling active neurons would have identified which neurons possess vigor-related activity. Even though limitations prevented the proposed study from being performed the previously mentioned methods acted to simulate the process of investigating the research questions, albeit by different means. The previously collected behavioral data and neural imaging from Dr. Gahtan's laboratory were analyzed to demonstrate what potential results may have been found. This method was not without its limitations, but it provided data that could be analyzed and interpreted to provide information about the process of movement vigor and the shared similarities between humans and zebrafish. Future work could incorporate similar methods to identify and improve specific PD symptomatology. PD patients suffering from decreased dopaminergic activity experience a variety of symptoms. Being able to

pinpoint specific neuron clusters will allow novel treatment options to be developed targeting those exact neurons. In theory, targeting vigor-related neurons should improve the symptomology of motor impairments if we are able to identify which neurons are active during different stages of motor functions and by determining what role they play. The implication of this research can provide support for the adoption of the movement vigor hypothesis and the process of targeting specific neuron clusters to treat PD symptoms.

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