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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Parkinson's disease (PD) poses one of the greatest healthcare challenges of our time. With the aging of the general population, the incidence of PD is expected to rise in coming decades. This wave will create not only a humanitarian crisis, but an unsustainable tax on societal resources. Although there are effective symptomatic therapies for the motor symptoms of PD (at least in the early stages of the disease), there are not effective therapies for the non-motor, PD and the commonly co-morbid, Lewy body dementia (LBD). This unmet medical need is identified as one of the FY19 PRP Focus Areas - Mechanisms of non-motor symptoms of PD from basic biology to clinical application. This gap in clinical care reflects our poor grasp of disease mechanisms. While it is widely accepted that mitochondrial dysfunction, synucleinopathy and inflammation contribute to PD and LBD, it is far from clear why these disease mechanisms manifest themselves in some neuronal populations and not others. All neurons rely upon proper protein handling. All neurons depend upon mitochondrial function. All neurons appear to be susceptible to the production of inflammatory cytokines and reactive oxygen species by non-neuronal cells. Understanding the basis for this selective vulnerability could provide the insight needed to develop new, potent therapies for nonmotor symptoms in PD. One of the most vulnerable types of neuron in PD, LBD and Alzheimer's disease (AD) is the basal for brain cholinergic neuron (BFCN). Release of acetylcholine (ACh) by BFCNs modulates the activity of large cortical networks, and the degeneration of these neurons is widely thought to be a primary driver of the cognitive deficits accompanying PD, LBD and AD. Yet, very little is known about how or why these neurons should be vulnerable to mitochondrial dysfunction, synucleinopathy or inflammation.

15. SUBJECT TERMS None listed.					
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1. Introduction:

Parkinson's disease (PD) and Lewy Bodies dementia (LBD) pose a major healthcare challenge affecting millions of people worldwide. Since age is a primary risk factor for both these conditions, the incidence is projected to steadily rise along with the increase longevity of the population. The absence of disease-modifying therapies and effective symptomatic treatments originate from our poor understanding of the mechanisms driving pathogenesis in PD and LBD. Hallmarks of PD and LBD are synucleinopathy, mitochondrial dysfunction and inflammation. However, how these changes are linked to selective neuronal disfunction is poorly understood. BFCNs are among the most vulnerable neurons in PD and LBD and their degeneration is thought to be responsible for the non-motor cognitive dysfunction in patients. This grant application is designed to begin understand some of the intrinsic and extrinsic determinant of BFNCs vulnerability in the context of synucleinopathies.

2. Keywords:

Dementia, Alzheimer's disease, Aging, metabolic syndrome, metabolism regulation, cholinergic, bioenergetics, Agnosia, Anomia, Frontotemporal dementia, vascular dementia, sleep/wake, dysphagia.

3. Accomplishments:

What were the major goals of the project?

Our primary research goal is to systematically attack this question using an array of newly developed methodologies that provide an unprecedented capacity to rigorously characterize the genetic, bioenergetic, physiological and anatomical determinants of selective neuronal vulnerability**.** With these powerful tools in-hand, we propose to pursue three specific aims:

Specific Aim 1: *To characterize how autonomous and synaptically-driven activity is generated in BFCNs.* A primary driver of vulnerability is likely to lie in the physiological phenotype of BFCNs – that is, in the traits required for them to fulfill their role in coordinating the activity of large scale cortical and hippocampal networks. The proposed studies employ an advanced array of electrophysiological and optical approaches in conjunction with anatomical methods to characterize two key types of BFCNs in transgenic mice. To provide a molecular anchor to this functional analysis, RiboTag and single-cell RNA harvesting methods will be used in conjunction with RNASeq and quantitative polymerase chain reaction (qPCR) approaches to characterize BFCNs.

Specific Aim 2: *To characterize the relationship between regenerative activity and bioenergetic control in BFCNs*. Our working hypothesis is that the combination of sustained regenerative activity and a massive axonal arbor elevates bioenergetic demand in BFCNs, resulting in sustained mitochondrial oxidant stress that increases synuclein misfolding and susceptibility to inflammation, particularly with advanced age. To test this hypothesis, an array of electrophysiological, optical and genetic strategies will be employed to study the bioenergetic control mechanisms and resulting oxidant stress in somatodendritic and axonal regions of BFCNs from transgenic mice.

Specific Aim 3: To determine the consequences of local and regional synucleinopathy on BFCNs. A hallmark of PD and LBD is the accumulation of misfolded forms of alpha-synuclein (αSYN). Our working hypothesis is that synucleinopathy engages both cell autonomous and non-autonomous (extrinsic) mechanisms to induce BFCN degeneration in PD and LBD. As a first step toward testing this hypothesis, αSYN pre-formed fibrils will be stereotaxically introduced and the functional impact on BFCNs determined using a combination of electrophysiological and optical approaches in transgenic mice. These studies will provide the first clear assessment of αSYN-induced pathophysiology in BFCNs and in so doing should point to strategies for mitigating it.

What was accomplished under these goals?

Specific Aim 1: *To characterize how autonomous and synaptically-driven activity is generated in BFCNs.*

Determinants of BFCNs pacemaking activity

In other neurons, autonomous pacemaking is regulated by the interaction between depolarizing cation channels and voltage-dependent K^+ channels. Two complementary approaches were used to characterize

these mechanisms in BFCNs. First, the transcriptomes of BFCNs were determined by harvestings actively transcribed mRNAs from BFCNs using the RiboTag approach and then performing RNASeq. Second, BFCNs were subjected to patch clamp analysis in ex vivo brain slices.

Transcriptomic analysis revealed that BFCNs express a wide variety of voltage-dependent K^+ channels (in order of abundance): **Kv4** (Kv4.3 > Kv4.2 > Kv4.1), **Kv1** (Kv1.2 > Kv1.6 > Kv1.1 > Kv1.4 > Kv1.3 > Kv1.5), **Kv3** (Kv3.2 > Kv3.1 > Kv 3.4 > Kv3.3), and **Kv2** (Kv2.2>Kv2.1). Both Kv4 and Kv1 channels are sensitive to 4-aminopyridine (4-AP). At lower concentrations (100 µM), 4-AP preferentially blocks Kv1 channels while at higher concentrations 4-AP also blocks Kv4 channels. In cell-attached recordings, Kv1-selective concentrations of 4-AP increased spiking rate (**Figure 1A,B**). Higher concentrations further increased the spiking rate (**Figure 1B,C**), suggesting that Kv1 and Kv4 channels negatively regulated autonomous spiking in BFCNs – in agreement with the transcriptomic analysis.

Two cation channels commonly contribute to the inward current necessary to sustain pacemaking: HCN and NALCN channels. Whole cell patch clamp recordings of BFCNs has failed to find any evidence of prominent HCN channel currents - see figure 1 grant application and (Unal *et al.*, 2012). On the other hand, our transcriptomic analysis revealed that NALCN channels are robustly (?) expressed by BFCNs. Unfortunately, the pharmacological tools available to test the functional role of these channels are not well-validated. Recent work has suggested that the N-benzhydryl quinuclidine (L-703,606) inhibits NALCN channels (Hahn *et al.*, 2020). However, L-703,606 did not alter BFCNs basal firing rate, suggesting that either this compound does not inhibit all NALCN channels or they are not involved in autonomous spike generation in BFCNs. Another cation channel implicated in autonomous spiking is the transient receptor potential cation channel, subfamily C, member 3 (TRPC3). This is a sub-family of channels that is known to regulate the activity of striatal cholinergic interneurons, and our transcriptomic analysis shows, indeed, that BFCNs express TRPC3 channels. However, selective pharmacological tools to study TRPC3 are lacking. Going forward, our plan is to use genetic approaches (e.g., viral delivery of short-hairpin RNAs) to knockdown the expression of NALCN and TRPC3 channels to determine their role in autonomous pacemaking.

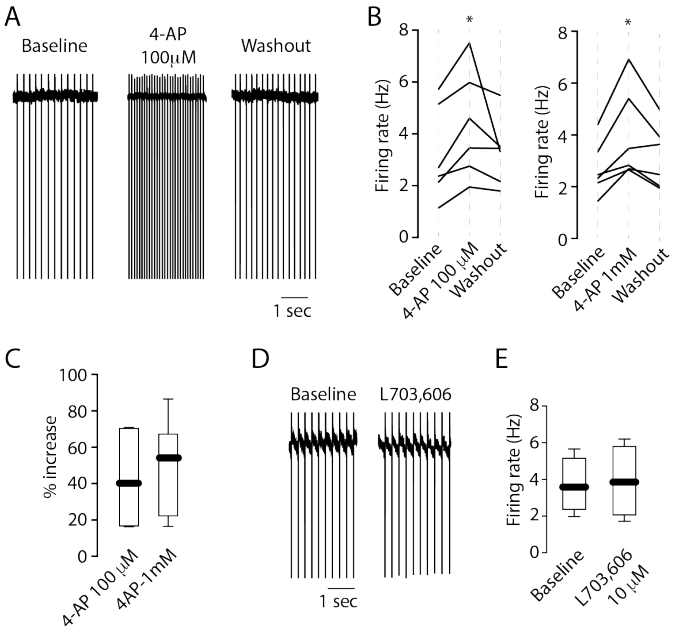

Figure 1. Kv1 channels modulate BFCN spiking rate. A, Representative traces showing the increase in firing rate obtained with bath application of 100 µM 4-AP. **B,** graphs summarizing the effect of 4-AP 100 µM and 1 mM on firing activity. Both concentrations produce the same increase in firing rate, **C**. **D,** Representative traces of BFCNs spiking activity before and during L703,606 application. No significant effect of this drug was noted, **E**.

Synaptic determinants of BFCNs activity

Previous anatomical (Gielow & Zaborszky, 2017) studies have shown that the most robust innervation of BFCNs in the substantia innominata/nucleus basalis magnocellularis (SI/NB) arises from the ventral striatum (vStr). In the vStr, there are two types of GABAergic spiny projection neuron (SPN). One that expresses D1 dopamine receptors (D1SPNs) and another that expresses D2 dopamine receptors (D2SPNs). Previous work has not distinguished between these projection phenotypes. Our preliminary studies show that both D1SPNs and D2SPNs project to SI/NB, but their innervation density appears to be quite different. Confocal imaging of the BF in Drd1a-tdTomatotg/Drd2-EGFPtg BAC transgenic mice where D1SPNs are labelled in red and D2SPNs are labelled in green suggest that D1SPNs provide a much stronger innervation of the BF than do D2SPNs (**Figure 2**).

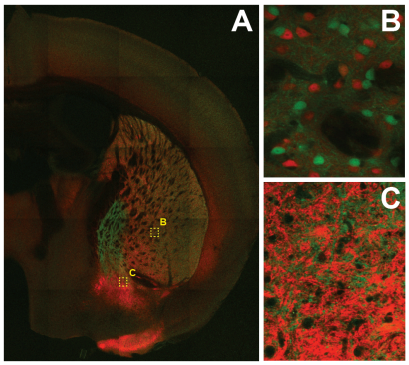

Figure 2. A, Confocal image of a slice from a Drd1a-tdTomatotg/Drd2-EGFPtg BAC transgenic mice dSPNs labelled in red (tomato) and iSPNs labelled in Green (GFP). **B, C,** magnification of the striatal (**B**) and BF (**C**) regions.

We are now rigorously pursuing these observations using optogenetic and physiological approaches. As a first step, the opsin Chronos was expressed in vStr SPNs and identified BFCNs recorded using cell-attached methods (**Figure 3**). As expected

of a GABAergic projection, optogenetic activation of SPN axons suppressed autonomous pacemaking in BFCNs (**Figure 3B top**). Cell-attached recordings were used in these experiments to allow the naturally occurring Cl- gradients to determine the polarity of the response to synaptic stimulation. In these initial experiments, the opsin was expressed in both D1SPNs and D2SPNs. However, the response of BFCNs had a feature that was consistent with a major role of D1SPNs in this projection – as inferred from the anatomical analysis (Figure 2). In addition to releasing GABA, SPNs release peptide neuromodulators. D1SPNs release substance P (SP) and D2SPNs release enkephalin (Enk). SP activates neurokinin 1 receptors (NK1Rs) that depolarize neurons, whereas Enk activates delta or mu opioid receptors to hyperpolarize neurons. Our transcriptomic analysis revealed that BFCNs robustly express NK1Rs, but have only modest expression of delta1 and mu1 opioid receptors. Consistent with this profile, optical stimulation of SPN axons in the presence of $GABA_A$ and $GABA_B$ receptor antagonists revealed a slow excitation of BFCNs that was consistent with SP release and NK1R activation (**Figure 3B bottom trace**). In agreement, bath application of SP (1 μM) increased BFCN spiking rate (data not shown).

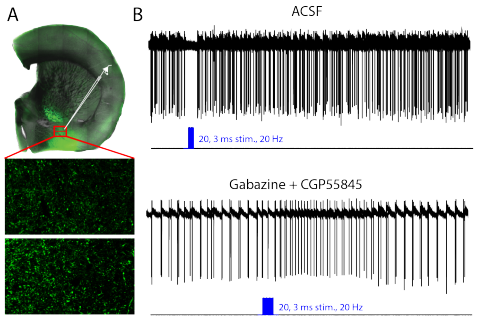

Figure 3. Cell-attached recording performed in BFCNs from Ai14 x ChAT-cre mice stereotaxically injected with AAV-hSyn-Chronos-eGFP in vStr. **A,** Expression of the opsin reporter is high in the SPN axons invading the BF (A, lower images). **B,** Optical stimulation (3 ms stimulation duration delivered 20 times at 20 Hz) obtained with an 470nm LED transiently stopped BFCN spiking (**B,** upper trace). Bath application of $GABA_A$ and $GABA_B$ receptor antagonists (Gabazine and CGP55845 respectively) uncovered a delayed excitatory effect elicited using the same stimulation

Transcriptomic studies of parvalbumin-positive GABAergic neurons in BF

As noted above, our transcriptomic analysis of BFCNs has proceeded as expected. However, our analysis of parvalbumin-expressing (PV+) neurons encountered a problem. PV+ neurons are intermingled with BFCNs in the BF and have a similar axonal projection fields. In contrast to BFCNs, these neurons are relatively spared in PDD and other dementias. Therefore, a comparison of BFCNs and PV+ transcriptome might be particularly informative, as it could reveal determinants of their differential vulnerability. These studies have been delayed by problems with our PV-cre recombinase mouse line that would be used to express RiboTag just in this population. We discovered that the expression of cre recombinase was unstable. Therefore, we imported a new line of PV+-cre recombinase mice and have established a breeding colony. We plan to perform the experiments proposed within the next six months.

Progress for Specific Aim 2: *To characterize the relationship between regenerative activity and bioenergetic control in BFCNs*.

Mechanisms coupling spiking to mitochondrial oxidative phosphorylation in BFCNs

Our studies suggest that BFCNs engage a Ca^{2+} dependent feed-forward mechanism couples spiking to mitochondrial ATP production (**Figure 4A**). Although this feedforward mechanism ensures that the bioenergetic demands associated with spiking are met, this mechanism also can overwhelm anti-oxidant defenses and result in oxidant stress. Transcriptome analysis of BFCNs revealed the presence of all the elements thought to participate in this control system: Cav1 Ca2+ channel subunits, ryanodine receptors (RyR1-3), and the mitochondrial calcium uniporter (MCU). Physiological studies also are consistent with this mechanism. Specifically, using a mitochondrially-targeted redox sensor and two photon laser scanning microscopy in ex vivo brain slices, we have found that inhibition of Cav1 channels with isradipine or inhibition of RYRs with 1,1'-diheptyl-4,4'-bipyridinium dibromide (DHBP) lowers mitochondrial oxidant stress in BFCNs (**Figure 4B**). Consistent with the coupling of spiking to mitochondrial control, stopping pacemaking with the Nav1 channel blocker tetrodotoxin (TTX) also lowers mitochondrial oxidative stress (**Figure 4B**). These data support the view that BFCNs utilize a feedforward mechanism to

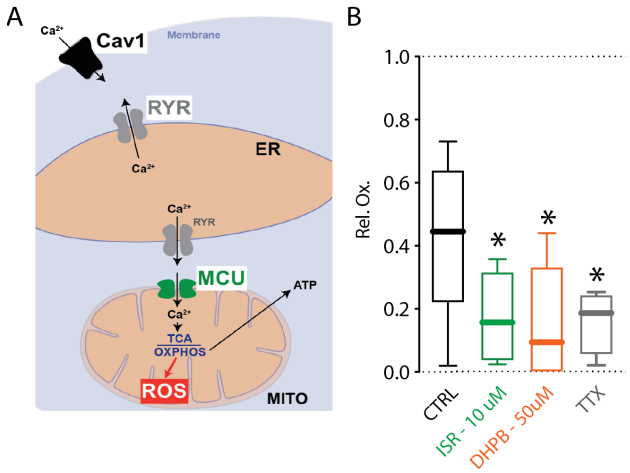

Figure 4. A, Relative oxidation data collected using a Cre-dependent mito-roGFP construct (insert) and ChAT-cre mice. One-hour pre-incubation of slices with Cav1 modulator isradipine (ISR.), the RYR modulator DHBP or TTX significantly reduced basal BFCNs mitochondrial oxidation. Old mice (* $p < 0.05$). **B,** Schematic representation of the intracellular Ca^{2+} pathway involved in feed-forward stimulation of OXPHOS and ATP production and ultimately increase in ROS.

control mitochondrial oxidative phosphorylation (OXPHOS). To complement these studies, we are exploring the potential role of the malate-aspartate shuttle in the joint regulation of glycolysis and OXPHOS.

Progress for Specific Aim 3: *To determine the consequences of local and regional synucleinopathy on BFCNs.*

Selective neuronal vulnerability in the BF to misfolded alpha-synuclein

A hallmark of dementia is the formation of alpha-synuclein (aSYN) containing Lewy pathology (LP) in the BF. These cytoplasmic inclusions are thought to contribute to neuronal dysfunction and death. To gain a better understanding of how LP affects BFCNs, we injected pre-formed fibrils of aSYN (PFFs) into the BF. To our surprise, aSYN pathology as assessed by S129 phosphorylation of aSYN was not present in BFCNs following PFF injection. One possible explanation for this result (provided by our transcriptome analysis) is that BFCNs don't express synphilin-1 (encoded by the gene SNCAIP). Previous work suggests that synphilin-1 is necessary for aSYN aggregate formation (Engelender *et al.*, 1999). We are investigating this lead by ectopically expressing a synphilin-1 in BFCNs prior to PFF injection.

One of the questions that remains to be answered is which cell type manifest aSYN pathology following PFF injection. Using BAC transgenic mice where PV+ neurons express tdTomato (PV-tdTomato) mice, we have determined that aSYN pathology is not present in PV+ GABAergic neurons in BF (**Figure 5**). The third population of neurons in the BF are glutamatergic neurons that express the vesicular glutamate transporter, subtype 2 (vGluT2). We have acquired VGLUT2-cre recombinase mice that will allow us to determine whether they manifest aSYN pathology following PFF injection.

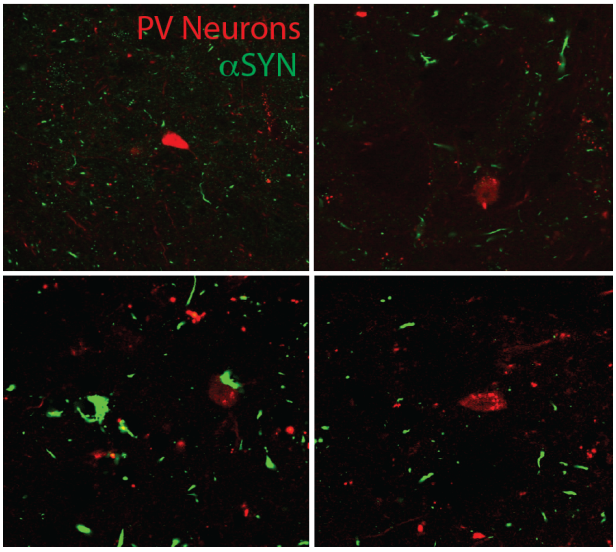

Figure 5. Accumulation of synuclein pathology in was not found in PV+ neurons following PFF injection. Slices from PV-tdTomato mice were immunostained with antibody against S129 phosphosynuclein (p-αSyn). Accumulation of S129 p-αSyn immunoreactivity (green) is not found in PV+ neurons.

Physiological effects of aSYN pathology on BFCNs

Although BFCNs do not manifest aSYN pathology following local injection of PFFs, their physiology is altered. Both basal spiking rate and mitochondrial oxidant stress are elevated. The elevation in mitochondrial oxidant stress is partially reversed by preincubating the slices with isradipine (ISR) and further lowered with the anti-oxidant N-acetyl cysteine (NAC) (**Figure 6**). Our working hypothesis is that PFFs induce a local inflammation and that astrocytes or microglia release reactive oxygen species (ROS). Kv4 (and possibly Kv1) channels that control basal spiking rate (see above) are redox sensitive and decrease their opening with oxidant stress (Subramaniam *et al.*, 2014). This could provide a simple explanation for the elevation in spiking rate. The elevation in spiking rate, opening of Cav1 Ca^{2+} channels and engagement of feedforward signaling are then likely to be responsible for the elevation in mitochondrial oxidant stress. These hypotheses are now being tested.

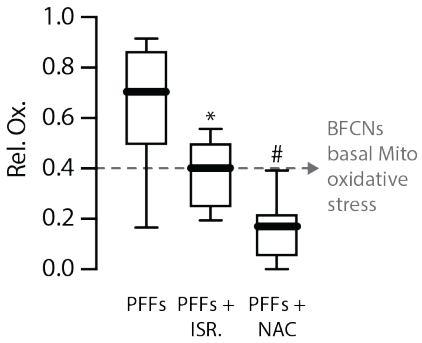

Figure 6. Isradipine (ISR.) preincubation partially reduces PFFs-mediated exacerbation of mitochondrial oxidation in BFCNs. NAC reduced mitochondrial oxidative stress to levels significantly lower than basal.

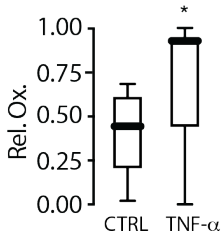

Figure 7. Pre-incubation of slices with TNFα for 30- 60 min increases basal BFCNs mitochondrial oxidation.

As mentioned above, PFF injection into the BF is accompanied by robust inflammatory response and astrocytic activation as evidenced by increased GFAP staining (data not shown). One of the inflammatory cytokines released by activated astrocytes is tumor necrosis factor α (TNF-α) (Chung & Benveniste, 1990; Olmos & Llado, 2014). TNF-α stimulates ROS production by several mechanisms. Indeed, pre-incubation of BF slices with TNFα directly increases mitochondrial oxidative stress in BFCNs (**Figure 7**). We also have found that lactate, which plays an important role in

gliotransmission, can significantly increase BFCNs firing rate (not shown). In agreement with these observations, optogenetic activation of astrocytes increases BFCN spiking (data not shown). Taken together, these data suggest that aSYN drives BFCN spiking and mitochondrial oxidant stress by promoting gliotransmission mediated by lactate and inflammatory cytokines. This hypothesis is now being rigorously tested.

References:

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What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
Our plan is to continue with the research plan outlined in the original proposal.

4. Impact:

What was the impact on the development of the principal discipline(s) of the project?
Although promising, our results have not had an impact on the neuroscience field yet. We are hoping that in the next award period this situation will change.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

5. Changes/Problems:

Changes in approach and reasons for change
We have not encountered any significant problems other that poor expression of Cre recombinase in the PV transgenic mouse, which has been replaced.

Actual or anticipated problems or delays and actions or plans to resolve them
The PV-Cre recombinase transgenic mice have been replaced.

Changes that had a significant impact on expenditures
None

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
None

Significant changes in use or care of human subjects
Not applicable.

Significant changes in use or care of vertebrate animals.
None

Significant changes in use of biohazards and/or select agents
Not applicable.

6. Products:

Publications, conference papers, and presentations
Nothing to Report

Journal publications.
Nothing to Report

Books or other non-periodical, one-time publications.
Nothing to Report

Other publications, conference papers, and presentations.
Nothing to Report

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
Nothing to Report

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	D. James Surmeier, PhD
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2
Contribution to Project:	Oversees the carrying out of the research plan to ensure all aims and goals are met. Works closely with the investigative team to

	develop experimental plans that test the proposed hypothesis, analyzes data from experiments, and leads the overall manuscript development process.

Name:	Tristano Pancani, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-0511-7702
Nearest person month worked:	8
Contribution to Project:	Responsible for the conduct of the work proposed in Specific Aim 2, part of Specific Aim 3.

Name:	Tatiana Tkatch, PhD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5
Contribution to Project:	Responsible for the conduct of Specific Aim 1 and portions of Specific Aim 3.

Name:	Jaime Guzman-Lucero, PHD
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-1746-8537
Nearest person month worked:	4
Contribution to Project:	Responsible for assisting with the experiments in Specific Aim 2 and for conducting the experiments outlined in Specific Aim 3. He assists with experimental design, data analysis, manuscript development, and works closely with the rest of the investigative team.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

New active awards:

- NIH, R37 NS034696, PI: Surmeier, began 7/1/20, effort 1.1 CM
- NIH, R01 NS121174, PI: Surmeier, began 4/15/21, effort 1.38 CM
- NIH via University of Texas Southwestern, GMO210305 PO
  PU: Dauer,began 2/1/21, effort 0.96 CM

Completed awards:

- NIH, R01 NS034696, PI: Surmeier, ended 6/30/20
- Michael J. Fox Foundation, Grant# 15031, PI: Surmeier, ended 10/31/20
- Michael J. Fox Foundation, Grant# 15031.01, PI: Surmeier, ended 4/16/221

What other organizations were involved as partners?
Nothing to Report.

8. **Special Reporting Requirements**
   Attached.

9. **Appendices:**
   Not applicable.