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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.

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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.*

Intrinsic and extrinsic determinants of pacemaking activity. RNASeq analysis of RiboTag isolated mRNA from basal forebrain cholinergic neurons (BFCNs) revealed the presence of transcripts for a variety of voltage-dependent K^+ channels including Kv4, Kv3, Kv2 and Kv1. Many of these channels have been implicated in the regulation of autonomous pacemaking. Many of these channels are redox sensitive, coupling intracellular signaling through reactive oxygen species (ROS) and nitric oxide (NO) to membrane excitability and spiking rate (Sahoo *et al.*, 2014). Both our transcriptomic analysis and immunocytochemical localization of protein demonstrate that most (~60-70%) BFCNs express neuronal nitric oxide (nNOS; **Fig. 1**). The abundance of nNOS mRNA is significantly higher in BFCNs than in substantia nigra pars compacta dopaminergic neurons (DAs), pedunculopontine cholinergic neurons (PPNs) or striatal cholinergic interneurons (transcriptome analysis, not shown). Consistent with previous work showing that redox-mediated down-regulation of K^+ channel function, blocking nNOS activity with L-NAME for 30-60 minutes significantly reduced BFCNs spiking activity (**Fig. 2**), suggesting that tonic NO signaling enhances basal spiking rate. These experiments will be confirmed with a selective nNOS inhibitor developed by Dr. Silverman at Northwestern University.

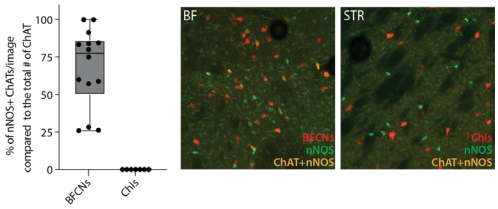

Fig. 1. BFCNs but not striatal cholinergic interneurons (ChIs,) express nNOS (green) ChAT-expressing neurons in red. In orange the overlap ChAT with nNOS.

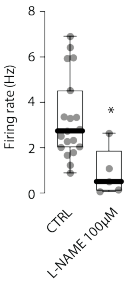

Fig. 2. Preincubation of acute slices with the non-selective NOS inhibitor L-NAME reduces BFCNs firing rate. Mann-Whitney non-parametric test * $p<0.05$

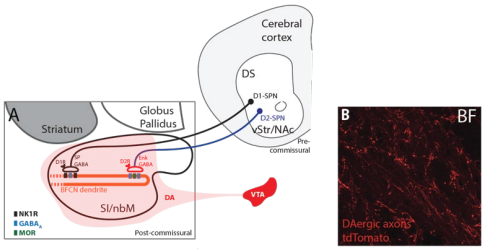

Fig. 3. A, Schematic showing of SPNs-BFCNs network. SPNs in the precommissural ventral striatum (vStr)/Nucleous Accumbens (NAc) send projection to the post-commissural basal forebrain: substantia innominata/nucleous basalis magnocellularis (SI/nbM); D1-SPN express D1 dopamine (DA) receptors and release GABA and substance P (SP) onto BFCNs dendrites; D2-SPNs express D2 DA receptors and release GABA and Enkephalin (Enk) on the BFCNs dendrites. **B,** Axons expressing tdTomato fluorescence from Ventral tegmental area (VTA) provides the main DAergic innervation to the basal forebrain (BF).

Extrinsic determinants of spiking activity. Retrograde tracing has shown that GABAergic striatal spiny projection neurons (SPNs) provide a very robust innervation of BFCNs (Gielow & Zaborszky, 2017). Both D1 dopamine receptor (D1R) expressing SPNs and D2 dopamine receptor (D2R) expressing SPNs contribute to this innervation (**Fig. 3**). Stimulation of GABAergic SPN axons in ex vivo brain slices of the BF causes a pause in the basal spiking of BFCNs monitored in the cell-attached recording configuration. After this pause, there is a period of accelerated spiking (**Fig. 4**). Our working hypothesis is that this acceleration is mediated by the release of substance P (SP) by D1R-expressing SPNs, which activates postsynaptic NK1 receptors (NK1Rs) that are robustly expressed by BFCNs (as revealed by the RNASeq data). One potential modulator of this synapse is dopamine. Indeed, dopaminergic neurons in the ventral tegmental area (VTA) innervate the BF (**Fig. 3A**). In principle, dopamine release by these neurons should increase GABA and SP release by terminals arising from D1R-expressing SPNs. Consistent with this hypothesis, bath application of the D1R agonist SKF 81297, enhanced the acceleration of spiking rate in BFCNs following stimulation of SPN axons, but did nothing on its own (**Fig. 5**). To more rigorously characterize this circuit, mice expressing Cre recombinase selectively in D1R-SPNs have been crossed with mice expressing eGFP in cholinergic neurons (D1-cre x ChAT-eGFP); optogenetic and electrophysiological techniques will be used in brain slice from these mice to characterize the functional connectivity of SPNs with BFCNs and to determine how it is modulated by VTA dopaminergic neurons.

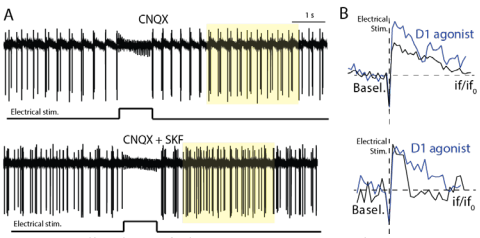

Fig. 4. Application of the D1 receptor agonist SKF81297 enhanced the post-stimulation increase in firing rate. Traces obtained in cell-attach configuration (blue traces in B).

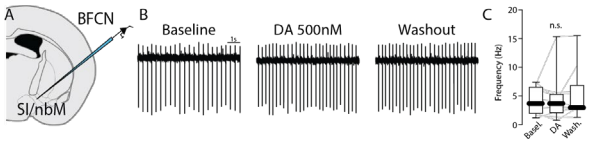

Fig. 5. Bath application of dopamine (DA) 500nM did not change basal firing rate of BFCNs. Traces obtained in cell-attach configuration

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

Our work to date suggests that BFCNs engage a Ca^{2+} -dependent, feed-forward mechanism to couple neuronal activity with mitochondrial ATP production. This type of mechanism is used by substantia nigra (SN) dopaminergic neurons, which like BFCNs spike autonomously and have robust intracellular Ca^{2+} transients that are phase-locked to spiking. SN dopaminergic neurons rely upon this feedforward mitochondrial control to generate the ATP needed to meet their bioenergetic needs (Gonzalez-Rodriguez *et al.*, 2021)(Zampese et al., in review at Science Advances). Despite these phenomenological similarities, BFCNs appear to rely much more upon glycolysis than mitochondrial oxidative phosphorylation to meet there bioenergetic needs. Our working hypothesis is that this difference reflects the need in BFCNs to "highjack" mitochondria to produce acetylcholine (from citrate in the tricarboxylic acid cycle). Indeed, the comparative RNASeq analysis showed that the expression of many if not all of the genes related to glycolysis was higher in BFCNs than in SN dopaminergic neurons.

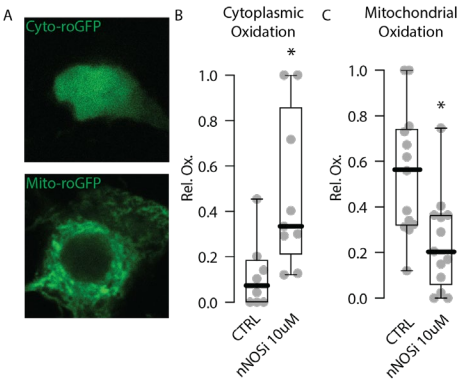

Fig. 6. A, Representative fluorescence images of BFCNs expressing a cytoplasmic targeted roGFP probe (top) and a mitochondrially targeted roGFP construct (bottom). Pre-incubation of acute slices with a selective nNOS inhibitor increases cytoplasmic oxidative stress (**B**) and reduces mitochondrial oxidative stress (**C**)

If the cytosolic Ca^{2+} transients linked to pacemaking are not being used to drive mitochondrial oxidative phosphorylation, what is their function? As mentioned above, BFCNs express high levels of Ca^{2+} stimulated nNOS. NO inhibits mitochondrial complexes I and IV, slowing oxidative phosphorylation and increasing oxidant stress. Indeed, inhibiting nNOS in BFCNs lowers mitochondrial oxidant stress (**Fig. 6C**). In this way, NO may promote glycolytic ATP production and activity in the pentose phosphate shunt, which helps to maintain cytosolic redox balance. Consistent with this hypothesis, inhibiting nNOS significantly increases cytosolic oxidant stress in BFCNs (**Fig. 6B**). Interestingly, inhibition of nNOS also appears to lower activation of AMP kinase (AMPK) – a key regulator of cellular bioenergetics (**Fig. 7**). A major goal in the next award period will be to more clearly define how these mechanisms work together to control bioenergetics in BFCNs.

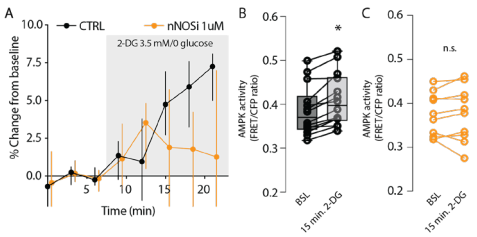

Fig. 7. A, Graph showing that perfusing the slices with ACSF modified with 2-DG and 0 glucose for 15 minutes increases AMPK activity (black, **B**). Pretreating the slices with nNOS inhibitor reduces significantly this effect (orange and **C**).

As this effort moves forward with the incorporation of genetically encoded optical sensors, we are keenly aware of the implications this regulatory system may have for the response of BFCNs to local inflammatory processes, like those engaged by synucleinopathy. NO and ROS are generated by activated microglia. These exogenous signals might dysregulate BFCN bioenergetics and ACh synthesis. To pursue this possibility (as detailed below), brain microglia are being depleted by feeding mice the colony-stimulating factor-1 (CSF1R) inhibitor PLX5622. Our prediction is that doing so will blunt the changes in BFCN bioenergetics induced by local injection of alpha-synuclein pre-formed fibrils – a potent activator of local microglia.

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

Physiological effects of aSYN pathology on BFCNs. The development of alpha-synuclein (aSYN)-containing cytoplasmic inclusions is thought to contribute to neuronal dysfunction and cell death in several parts of the brain, including the BF, in PD and Lewy body dementia. To determine the effects of misfolded aSYN on BFCNs, pre-formed fibrils of aSYN (PFFs) and monomeric aSYN was stereotaxically injected into the BF of mice. Twelve weeks later, mice were sacrificed, brains sectioned and processed for S129 phospho-aSYN immunoreactivity (a standard assay for aSYN pathology). Surprisingly, BFCNs had no discernible aSYN pathology, despite it being present in neighboring neurons and there being a clear elevation in microglial activation. Subsequently, we've determined that the aSYN pathology present is not present in parvalbumin-positive, GABAergic neurons, astrocytes or microglia; this result points to vGlut2-expressing glutamatergic neurons as the bearers of S129 immunoreactivity. Experiments to nail this conclusion down are underway.

Despite the absence of S129 aSYN, BFCNs are clearly affected by regional pathology. As reported earlier, PFF injection increases BFCNs firing rate significantly. As this effect is diminished by free radical scavengers, our working hypothesis is that it is attributable to ROS and/or NO released by activated microglia, which inhibit Kv4

and/or Kv1 K^+ channels controlling basal spiking rate. Inflammatory cytokines, like TNF-alpha (which we have found also increase BFCN spiking rate) may also contribute to this increase in spiking rate.

The metabolic consequences of the increase in BFCN discharge rate when challenged by PFFs remain to be determined. It is possible that increased Ca^{2+} entry accompanying the increased spiking amplifies feedforward activation of mitochondria oxidative phosphorylation. But this scenario seems inconsistent with the data presented above implicating cytosolic Ca^{2+} in nNOS activation, NO generation and inhibition of mitochondrial oxidative phosphorylation. To help sort out these possibilities, the RiboTag/RNASeq methodology described above has been employed to determine transcriptomic changes in BFCN following local injection of PFFs or monomeric aSYN. The initial analysis of this data has revealed that the expression of many mitochondrial genes is significantly increased in following aSYN PFF or monomer injection. This is in stark contrast to the transcriptomic changes in SN dopaminergic neurons following local PFF injection; in these neurons, which manifest S129 aSYN pathology, mitochondrial genes are strongly down-regulated. Further analysis of this data is underway to determine if there are other pathways that are up- or down-regulated by this perturbation. Lysosomal and proteostatic genes will be of particular interest.

Lastly, one of the most interesting observations in this set of experiments was that injection of monomeric aSYN into the BF had a qualitatively similar impact on the expression of mitochondrial genes as did PFF injection. Although there was no discernible S129 pathology following monomer injection, there may be other pathogenic effects. Overexpression of α-SYN monomers has been reported to impair mitochondrial function, increase oxidant stress and activate cell death pathways (Dettmer *et al.*, 2015; Visanji *et al.*, 2016; Lindstrom *et al.*, 2017). It is possible that despite the lack of S129 pathology, BFCNs are taking up aSYN monomers (and PFFs). This possibility will be explored in the near future by looking at markers of lysosomal and proteosomal function in BFCNs. In addition, signs of inflammation following monomer injection will be re-examined.

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Sahoo, N., Hoshi, T. & Heinemann, S.H. (2014) Oxidative modulation of voltage-gated potassium channels. *Antioxid Redox Signal*, **21**, 933-952.

Visanji, N.P., Brotchie, J.M., Kalia, L.V., Koprich, J.B., Tandon, A., Watts, J.C. & Lang, A.E. (2016) alpha-Synuclein-Based Animal Models of Parkinson's Disease: Challenges and Opportunities in a New Era. *Trends Neurosci*, **39**, 750-762.

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

How were the results disseminated to communities of interest?
Sissa (International School for Advanced Studies, Trieste, Italy), Neuroscience Seminars July 2021, Presentation “Cholinergic modulation of cortico-basal loops”

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?
Nothing to Report

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 do not plan on changing our approach.

Actual or anticipated problems or delays and actions or plans to resolve them
We do anticipate any significant problems or delays.

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 and Specific Aim 4.

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 4.

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:

- MJFF, MJFF-019948, PI: Surmeier, began 8/1/21, effort 0.12 CM
- Department of Army, W81XWH2110749, PI: Surmeier, began 9/30/21, effort 0.60 CM
- MJFF, MJFF-019886, PI: Chandel, began 9/1/21, effort 0.06 CM
- MJFF, ASAP-020551, PI: Surmeier, began 11/1/2021, effort 3.00 CM
- MJFF, ASAP-020600, PI Awatramani, began 11/1/2021, effort 0.37 CM
- JPB, GR-2021-2960, PI: Surmeier, began 11/1/2021, effort 0.60 CM
- NIH, R01NS099623, PI: Deng, began 12/2021, effort 0.06 CM
- MJFF, MJFF-021158, PI: Surmeier, began 2/1/2022, effort 0.06 CM

Completed awards:

- NIH, R01 NS099623, PI: Deng, ended 7/31/2021
- Department of Army, W81XWH1810778, PI: Surmeier, ended 9/29/2021

What other organizations were involved as partners?

Nothing to Report.

8. Special Reporting Requirements

Attached.

9. Appendices:

Not applicable.