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IN THE SHADOW OF JOHN BIENENSTOCK: TARGETING THE ENTERIC NERVOUS SYSTEM TO TREAT NEURODEGENERATIVE DISEASES

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INTRODUCTION

John Bienenstock has been colleague of mine for over a decade and with whom I have shared many inspiring discussions across the broad range of basic science and medicine. My contribution to the 2023 Old Herborn University Seminar focuses on our mutual research interests that have led to several successful clinical trials.

I first met John at a 2010 meeting of the Broad Research Foundation in Los Angeles at which recipients of grants awarded in the area of inflammatory bowel disease presented their work. At that meeting he presented data demonstrating that probiotic bacteria influenced behaviour/mood by stimulating signals within the enteric nervous system(ENS) that communicated to brain centres via the vagal nerves. The studies were subsequently published in 2011 (*Bravo* et al., 2011).

Ingestion of a *Lactobacillus* strain regulates emotional behaviour and central GABA receptor expression in a mouse via the vagus nerve). The basic theme was that microbes positioned within certain portions of the GI tract of the mouse could have a profound impact on central neurological symptoms. Most surprisingly, the effect of the bacteria appeared to depend on electrical signals transmitted from the intestine to the brain via the vagal nerves. These studies profoundly altered my understanding of the "Gut-Brain Axis." Previously I had assumed that the gut and brain communicated principally through circulating chemicals such as hormones or bioactive compounds. John's studies now extended the communication circuitry to electrical impulses between the ENS and the central nervous system (CNS).

PARKINSON'S DISEASE

Sometime later a family member of mine was diagnosed with Parkinson's disease (PD). This event led me to review the current understanding of the natural history and pathophysiology of this condition. In medical school, I was taught that PD first appeared clinically when the dopamine-rich neurons within the substantia nigra died off. Characteristic intra-neuronal inclusions called Lewy bodies were pathognomonic findings. I knew that dopamine replacement, achieved through the oral administration of L-DOPA, a dopamine prodrug, was a standard therapy. I had not realized that about 25 years ago a protein, alpha-synuclein (aS), had been linked to PD by genetic analysis of several rare families. Furthermore, aS was identified within the Lewy body and present in elevated amounts in both the peripheral nervous system and CNS of advanced cases of PD. Most curiously, I learned of the work of Braak, who had demonstrated through histology of the neural tissues of individuals

with PD ranging from the earliest symptoms of the disease to the most advanced, that aS inclusions appeared in the ENS many years before severe movement deficits were clinically evident. As the disease progressed aS accumulation appeared to progressively move, via the vagus, into the dorsal motor nucleus, and cranially, eventually reaching the substantia nigra. Braak hypothesized that PD actually begins in the ENS of the proximal GI tract through the accumulation of aS, which then is slowly transported cranially via the vagal nerves (*Braak* et al., 2003).

Braak's observation and hypothesis were supported by careful re-evaluation of the natural history of PD. Surprisingly, the vast majority of patients with PD experienced severe constipation many years before the onset of the classic signs and symptoms of PD. Indeed, several non-motor symptoms were identified that strongly predicted the eventual onset of PD, most robustly, REM behaviour sleep disorder (*Postuma* et al., 2012). Indeed, in the absence of other confounding medical conditions, isolated REM behaviour disorder is associated with a greater than 95% risk of developing PD (*Mahowald* and *Schenck*, 2013). In fact, upon questioning my family member I learned that he had suffered from both bowel issues and REM behaviour disorder for many years.

SQUALAMINE

As I read more deeply about aS, I learned that this 145-residue protein was unstructured when in aqueous solution and described as "intrinsically disordered." Due to the presence of several runs of lysine residues within the N-terminal portion of the protein, aS bound avidly to membranes that displayed negatively charged phospholipid headgroups (*Perni* et al., 2017). Upon binding the amino-terminal portion, aS adopted an alpha-helical configuration, while the carboxy-terminal portion remained free in solution. As the surface density of the monomeric aS molecules increased, closely positioned neighbours could aggregate forming membrane-active oligomers, which in turn could damage the physical integrity of the membrane (*Perni* et al., 2017). As it turned out, I had discovered a class of cationic sterols in the liver of the dogfish shark in the late 1990s and had been studying their biophysical properties as well as their pharmacology ever since (*Moore* et al., 1993; *Rao* et al., 2000). Indeed, one of these compounds, squalamine had entered several clinical trials for cancer and diabetic retinopathy some years earlier. Squalamine is a C27 bile salt in which the C24 hydroxyl is esterified with a sulphate, the C7 position contains an alpha-OH group, the C5 an alpha hydrogen, and the C3 position an alpha spermidine. The compound binds avidly to membrane surfaces composed of precisely the same phospholipids as does aS, and upon binding had been shown to displace positively charged peptides and proteins without disturbing the physical integrity of the membrane.

With colleagues at the NIH and Cambridge we showed that squalamine could displace aS from neuronal membranes, prevent aggregation, protect neuronal cells from lethal cytolysis caused by neurotoxic aggregates of aS (*Perni* et al., 2017). Exposure of a PD model of *C. elegans*, expressing a mutant aggregating form of aS, prevented both the formation of aS aggregates and the onset of paralysis in a dose-dependent fashion.

As these studies were progressing, I formed Enterin along with Dr. Denise

Barbut. Together we drafted the roadmap to the clinic with the intent of evaluating squalamine as a potential treatment for PD. Since we knew that most patients with PD suffered from constipation, and that aetiology of their dysmotility was likely due to accumulation of aS within the ENS, causing neuronal dysfunction, oral administration of squalamine might correct their constipation.

Most exciting, however, was the possibility, inspired by our many discussions with John and his colleague Wolf Kunze, that by restoring the electrical activity of the ENS we could restore communication between the ENS and the CNS. If, in fact, the aS-compromised ENS of the PD patient no longer communicated with brain, centres in the brain that normally received neuronal signals from the GI tract might atrophy, as occurs usually when an end organ is de-afferented. If so, as we corrected the constipation in PD patients, we might see improvements in other neurological symptoms.

PRECLINICAL STUDIES

With John and Wolf, along with Wolf's prior mentor, John Furness, we orally administered squalamine to mice engineered to express an aggregating form of human aS (*West* et al., 2020). Squalamine administration stimulated intestinal motility, as we had expected. Electrophysiological studies of the ENS, specifically the electrical activity of the intrinsic primary afferent neuron (IPAN), demonstrated that oral administration had restored their previously depressed excitability to normal, thus providing a mechanistic explanation for the improvement in intestinal motility.

In the next series of studies, we examined the impact of oral administration of squalamine in the PD mouse on the electrical signals directed to the brain via the vagal nerves (*West* et al., 2019). In these experiments single fibres were teased out of a branch of the mesenteric branch of the vagus, a parasympathetic stimulus was introduced onto the ENS, and the resulting electrical activity monitored with respect to amplitude, frequency and duration of excitation. We demonstrated that exposure of the ENS to squalamine dramatically stimulated the intensity and duration of signals directed from the ENS via the vagus to the brain.

From the time that John had shown that certain strains of *Lactobacillus* could induce a vagally transmitted signal that calmed mice he and Wolf believed that the electrical signals carried information. These signals were not "noise", but rather "music," characterized by specific patterns of frequency and amplitudes. John and Wolf described these patterns as the "SSRI" code, since their ongoing studies had demonstrated similarities between the signal patterns stimulated by the application of either the calming *Lactobacillus* strain or several selective serotonin reuptake inhibitors. The paper that describes John's thoughts was published in a series of experiments that included squalamine which, like the probiotic and an SSRI therapeutic, stimulated an SSRI-like vagal signal (*West* et al., 2021). Whole brain cFos imaging of mice following oral administration squalamine confirmed that extensive areas of the brain were either inhibited or activated by the compound. Since squalamine is not absorbed from the GI tract after oral administration, the effects observed result exclusively from communication between gut and brain.

CLINICAL TRIALS

In parallel with these studies, Enterin conducted two clinical trials, evaluating ENT-01 for the daily oral treatment of Parkinson's disease associated constipation over a 28-day period. (*Hauser* et, al., 2019). ENT-01 is a chemical stable crystalline phosphate salt of squalamine (which is why it was the preferred salt) that dissolves into the active squalamine zwitterion in the stomach. The first clinical trial, RASMET (https://clinicaltrials.gov/study/NCT03047629, was an open label study that evaluated the safety, tolerability, pharmacokinetics of ENT-01. In addition, we monitored effects on constipation as well as hallucinations and dementia in the subset of patients that had these symptoms. In addition, we monitored circadian rhythm and sleep parameters. As we reported, the RASMET trial demonstrated that ENT-01 corrected the constipation in a dose-dependent manner, very much reproducing the observations made with John and Wolf in the preclinical study. We also observed that in patients who had suffered from hallucinations, ENT-01 treatment had a dramatic effect, in some cases completely eliminating them for many weeks following cessation of treatment. Similarly in those patients suffering from dementia, significant improvement was observed during the 28-day treatment period. As in the case of hallucinations, cognitive functions continued to improve for several weeks after treatment had stopped. We monitored circadian rhythm through the measurement of skin temperature at the wrist using a wireless continuous recording device. Circadian rhythm is maintained through a complex circuit governed by the master clock within the hypothalamus, the suprachiasmatic nucleus. By monitoring circadian rhythm we can assess the "health" of the "beating" hypothalamus. Remarkably, the disturbed circadian rhythms of the untreated patients were corrected in a dose dependent fashion by oral administration of ENT-01. When we thought about it, after seeing the clinical data the effects on circadian rhythm made sense. The primary function of the circadian rhythm is to synchronize physical activity and hunger with the particular light/dark cycle appropriate for a species. Perhaps properly synchronized signals from the GI tract induced by a meal might be required to sustain a normal circadian rhythm. If so, by clearing the aS from the ENS, ENT-01 might be restoring necessary input to central circadian circuitry.

The RASMET clinical trial was followed by double blind randomized placebo-controlled Phase 2a study, KARMET (https://clinicaltrials.gov/study/NCT03781791. The study enrolled about 150 patients, each administered either placebo or ENT-01 for a period of 28 days. It successfully replicated the results of the RASMET trial. Constipation was corrected ($p<0.0001$), symptoms of both dementia and hallucinations were improved, and all effects of ENT-01 persisted for weeks after treatment ended. The study was published in the Annals of Internal Medicine (*Camilleri* et al., 2022). ENT-01 is now positioned to enter Phase 3 for PD associated constipation, Phase 2 for PD dementia, and Phase 2 for PD psychosis.

APPRECIATION

Both Denise and I have had the great pleasure and honour having worked with John and Wolf on the scientific journey I have described. John's

enthusiasm, the breadth of his knowledge of medicine and physiology made our collaboration intellectually exhilarating. If he disagreed with us, he told us directly, but was also ready to be corrected. This ability to converse freely in science, so smoothly and effortlessly is a rare characteristic and it made our friendship with John so very special. We miss him.

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