Organotypic hippocampal slices, an emerging tool to model synucleinopathies

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Parkinson's disease (PD) is the second most common neurodegenerative disease after Alzheimer's disease. PD has been traditionally considered a motoric disorder characterized by tremor, rigidity and bradykinesia, however it is now settled that PD also comprises a range of non-motor symptoms like hyposmia, sleep disturbances and cognitive impairments (Tysnes and Storstein, 2017).

The pathological hallmark of PD is the presence of neuronal cytoplasmic inclusions called Lewy bodies (LB) or Lewy neurites, which consist of the abundant presynaptic protein alpha-synuclein (α-syn) in an aggregated form. Apart from being present in all LB, α-syn is causally involved in rare cases of PD, where α-syn encoding *SNCA* gene either contains missense mutations or is dupli- or triplicated causing autosomal dominant familiar PD. Moreover, *SNCA* gene polymorphism is now believed to be a significant risk factor for the development of sporadic PD (Henderson et al., 2019).

Under normal condition, α-syn exists as native soluble form, but in PD, α-syn aggregates and accumulates as soluble oligomers and insoluble filaments in the diseased neurons. This process is governed by different factors related to ageing, genetics and environmental pollutants (Henderson et al., 2019).

Braak et al. (2003) formulated a hypothesis that suggests the spreading of α-syn aggregate pathology to and within the nervous system. Braak et al. (2003) proposed that the initial Lewy pathology starts at olfactory bulb or in the gut and then spreads to brain stem via vagal nerve, and this finding had been corroborated later by a large body of clinical and experimental evidences (Braak et al., 2003; Li et al., 2008, 2010).

In vitro studies have demonstrated that inoculation of recombinant preformed fibrils of α-syn (PFFs) or brain homogenates containing α-syn aggregates into the brains or peripheral tissue of animal models can initiate a progressive process of aggregation of endogenous α-syn. These newly formed aggregates are capable to spread between connected neurons triggering neuronal dysfunction and cell death. The biochemical and histopathological analysis of induced aggregates replicate the criteria of LB, namely ubiquitination and hyper phosphorylation (Delenclos et al., 2019).

Mechanistically, the available data support that α-syn aggregates, so-called seeds, are excreted from affected neurons and taken up by a neighboring healthy neuron where they template the aggregation of its native α-syn in a prion-like style. Hereafter, the process can replicate and spread further through the tissue. Evidences support that Braak's hypothesis is also applicable to other α-syn related diseases -collectively called synucleinopathies, e.g., dementia with LB and multiple system atrophy in different cellular and animal models in an anterograde or retrograde manner (Henderson et al., 2019). Furthermore, a recent *in vivo* study demonstrated that spreading of PFFs induced α-syn aggregates pathology can occur in both a retro- and anterograde manner upon inoculation of the seeds in the gut, replicating pathology observed in PD (Van Den Berge et al., 2019). So, to fully understand the mechanism of α-syn pathology spreading and progression, further investigations that reveal the molecular mechanisms of α-syn seeding, secretion, and how they are subsequently taken up by connected neurons where the progressive build-up of α-syn aggregates are continued are necessary.

Organotypic brain slices as a new model to study progressive a-syn pathology: Organotypic brain slice culture possess a potential of studying α-syn pathology and it's progression between connected neurons that is more faithful to the *in vivo* conditions than cell culture models. The slice cultures, still retain the advantages of *in vitro* systems compared to the more resource demanding conditions of animal experimentation. Stoppini et al. (1991) introduced a modified method for organotypic brain slice culture, where organotypic slices are maintained on a porous membrane interface between a humidified atmosphere and the culture medium. The use of membrane inserts preserves the cyto-architecture of the cultured brain region and this has resulted in a dramatic increased use of organotypic slices for studying electrophysiology, connectivity and drug screening (Croft et al., 2019). Since Stoppini et al. (1991) had introduced the interface method, organotypic slices had been driven and studied from different brain regions. Regions like ventral mesencephalon, striatum, hippocampus and cerebellum had been cultured either as single culture or co-cultures (Østergaard et al., 1990, 1991; Elfarrash et al., 2019; Shrivastava et al., 2020). Consequently, organotypic slices are considered as a feasible tool to address different questions in the field.

In our recently published work, organotypic hippocampal slices were optimized and characterized to model a progressive PD-associated pathology (Elfarrash et al., 2019). Microinjection of α-syn PFFs in the slices has successfully initiated the aggregation of endogenously expressed α-syn. Shrivastava et al. (2020) had also reported PFF induced aggregation in organotypic hippocampal slices using a different methodology where PFFs were diluted in fresh culture medium and applied on top of the slices. These findings suggest that the organotypic slice model can serve as an optimal bridging tool between the currently available *in vitro* models and animal models of PD.

Using organotypic slices allowed us to study neurons in a more physiologically optimal environment. In the slice culture, neurons are embedded in a glial matrix which is critical to replicate the *in vivo* environment, considering numbers of available data suggesting the active roles of microglia and astrocytes in the process of α-syn aggregates spreading and/or degradation (Ferreira and Romero-Ramos, 2018).

Organotypic hippocampal slices to study α-syn aggregates spreading: Using of organotypic hippocampal slices offers a simple unidirectional circuit of neuronal connections, where granule neurons of dentate gyrus (DG) are synaptically connected to pyramidal neurons of CA3 and subsequently to CA1 regions. This simple hippocampal circuit that is extensively used for electrophysiological recordings was found to be valuable to address the synaptic spreading of PFFs induced α-syn aggregates in the slice model. Microinjection of the PFFs at DG, allows the study of newly formed α-syn aggregates and the subsequent inter-neuronal spreading from DG to CA3 and CA1 (Elfarrash et al., 2019). This validates the usefulness of organotypic hippocampal slices as a novel *ex vivo* tool to address different controversial questions in the field, like the preference of antero- *versus* retro-grade spreading of α-syn aggregates pathology, ii) the influence of endogenous α-syn expression level for aggregates generation and iii) the role of phosphorylation of serine 129 in α-syn aggregation and spreading (Elfarrash et al., 2019). This surpasses previously used *in vitro* models that lack the neuronal connectivity of brain tissue and will simplify studying of mechanisms involved in formation, secretion, uptake and spreading of α-syn pathology in a more sophisticated but still easily manageable set-up.

The injection of PFFs at DG initiates the spreading of templated aggregate pathology via the preserved inter-neuronal connections of hippocampal slices to CA3 and CA1 regions in an anterograde manner. The process is relatively fast with spreading across two synapses from the DG via CA3 to CA1 pyramidal neurons accomplished in 7 to 10 days. At 14 days post-injection, LB-like aggregates were seen at CA1 region, corroborating a previous report suggesting CA1 pyramidal neurons to be more vulnerable for Lewy pathology formation in PD animal model (Luna et al., 2018).

The slice cultures allow for modulation of α-syn pathology as well. Expression of α-syn, mutant hereof or other proteins of interest can be easily manipulated by making slices from transgenic or gene knockout mice or via application of viral expression vectors. Alternatively, proteins of interest can be knocked down via using knockout mice or siRNA. The model opens for investigating functional effects of the progressive degeneration by combining it with live imaging of fluorescent reporter proteins, calcium imaging and even electrophysiological recordings.

As such, organotypic slices present an experimental model to both observe and manipulate the α-syn related pathology. This is essential to refine our understanding of the biological processes and mechanisms associated with the aggregates formation and spreading.

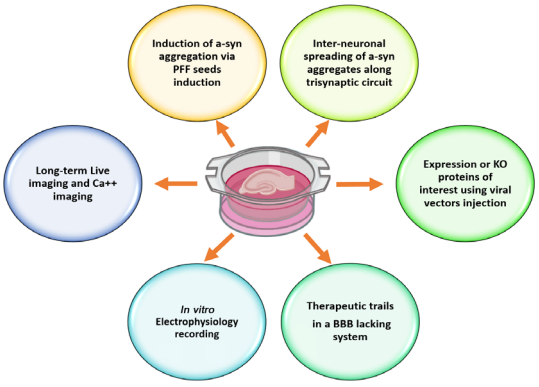

Figure 1 | **Potential applications of organotypic slices in the field of PD and other synucleinopathy related diseases.**
BBB: Blood-brain barrier; KO: knockout; PD: Parkinson's disease; PFF: preformed fibrils; α-syn: alpha-synuclein.

Studying post-translational modification (PTM) via combining slices with adeno-associated viral vector microinjection: Factors modulating the pathology, such as PTMs can be studied in the slice cultures. This is important because the role of PTMs like phosphorylation, ubiquitination, acetylation and sumoylation of α-syn are still controversial despite their potential as drug targets.

Organotypic slice culture offers a new platform to address PTM related questions. Different PTMs can be explored using different methods, such as using viral vectors, siRNA, or via direct application of specific kinase/s inhibitors for drug screening in a set up that lacks blood-brain barrier. The model will also replicate the physiological environment of the tissue where many kinases are expressed and located to specific subcellular sites. Importantly, as multiple slices are prepared from a single pup, this allows the investigation of several variables with a significant reduction of individual animal variations.

Despite phosphorylation at S129 (pS129) is considered as a hallmark of α-syn pathology, the available data on its mechanistic role are still conflicting (Oueslati, 2016). To reveal if phosphorylation at pS129 is essential for α-syn aggregates development or inter-neuronal spreading, organotypic slices from α-syn knock out pups were used to solely express a mutated α-syn (syn-S129G) that cannot be phosphorylated at Serine 129 (Elfarrash et al., 2019). Our data demonstrated that pS129 is not a prerequisite for aggregates formation or spreading in brain tissue. Moreover, this serves as a proof of principle for how the role of specific amino acids in the α-syn backbone can be easily addressed in slices.

Limitations of organotypic slice culture model: Naturally, organotypic slice model of synucleinopathy is not without limitations. Making and maintaining slices with minimal damage and with strict aseptic conditions is challenging, especially when interventions like microinjection of PFFs or viral vectors are needed. The fact that most of the organotypic slice cultures are reported to be favorably made from pups rather than adult mice or rats raises concerns about how well the slices can reflect the environment of mature or even aging brains. Emerging results suggest that organotypic slices can be made from adult brain - including human brain - when applying special conditions (Mewes et al., 2012). These studies still require further optimization and need to be replicated, as establishing a protocol which can maintain a large population of viable neurons in a slice made from adult mice will further promote the use of organotypic slices in synucleinopathy related research in the future.

Conclusion: Organotypic slice model of progressive PD neuropathology stands among other models with great advantages and perspectives. Maintaining three-dimensional tissue with preserved synaptic connections makes it superior to current *in vitro* models when studying the templated spreading of α-syn pathology. The simplicity and the possibility of combination with genetic modulation, drug screening, live imaging and electrophysiology will accelerate our understanding of synucleinopathies, and may help identifying and validate therapeutic targets in the near future (**Figure 1**). Organotypic slices can be seen as an alternative to some *in vivo* experiments. This will reduce both the number of animals required and the time needed to conduct some *in vivo* experiments.

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