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Fishing for synucleinopathy models

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Abstract

Synucleinopathies such as Parkinson's disease (PD) are incurable neurodegenerative conditions characterised by the abnormal aggregation of *α*-synuclein protein in neuronal cells. In PD, fibrillary synuclein aggregation forms Lewy bodies and Lewy neurites in the substantia nigra and cortex on the brain. Dementia with Lewy bodies and multiple system atrophy are also associated with *α*-synuclein protein abnormalities. *α*-synuclein is one of three synuclein proteins, and while its precise function is still unknown, one hypothesis posits that *α*-synuclein propagates from the enteric nervous system through the vagus nerve and into the brain, resulting in synucleinopathy. Studies on synucleinopathies should thus encompass not only the central nervous system but must necessarily include the gut and microbiome. The zebrafish (*Danio rerio*) is a well-established model for human neuronal pathologies and have been used in studies ranging from genetic models of hereditary disorders to neurotoxin-induced neurodegeneration as well as gut-brain-axis studies. There is significant genetic homology between zebrafish and mammalian vertebrates which is what makes the zebrafish so amenable to modelling human conditions but in the case of synucleinopathies, the zebrafish notably does not possess an *α*-synuclein homolog. Synuclein orthologs are present in the zebrafish however, and transgenic zebrafish that carry human *α*-synuclein have been generated. In addition, the zebrafish is a highly advantageous model and ideal replacement for reducing the use of mammalian models. This review discusses the application of the zebrafish as a model for synucleinopathies in efforts to further understand synuclein function and explore therapeutic strategies.

Keywords: Zebrafish, Synucleins, Synucleinopathy, Gut-brain-axis

Introduction

The synucleinopathies are distinct human diseases that share one commonality, the deposition of *α*-synuclein (SNCA) aggregations in the central nervous system (CNS). Synucleinopathies which include Parkinson's disease (PD), Parkinson disease dementia, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) feature chronic neurodegeneration of neurons that would have begun years before symptoms arise (Savica et al., 2018). PD is the most common synucleinopathy characterised by bradykinesia, resting tremor, rigidity, and postural and gait impairment – a collection of symptoms described as parkinsonism (Parkinson, 2002) – which develop due to the loss of dopamine and dopaminergic neurons in the substantia nigra parscompacta (SN) in the brain. Another hallmark feature of PD is the accumulation of SNCA Lewy bodies (LBs) throughout the CNS. As more LB deposits accumulate over time, parkinsonism symptoms worsen alongside progressive cogni-

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tive decline which presents as PD dementia. DLB is a type of dementia characterised by progressive and severe cognitive impairment along with parkinsonism, but as these symptoms are highly reminiscent of Alzheimer's Disease and PD, respectively, DLB is often misdiagnosed. Post-mortem confirmation of DLB would reveal widespread LB deposition in the brain. MSA is a rare, sporadic neurodegenerative disease that is characterised not only by parkinsonism but also autonomic failure that is far more severe compared to other synucleinopathies. Detection of glial cytoplasmic inclusions in the brain distinguishes MSA from other synucleinopathies (Coon & Singer, 2020; Dickson et al., 1999). These neurodegenerative diseases are devastating for the patients and their families, primarily impacting patients when they are in their golden years who then find themselves becoming dependent on others for their most basic and even private needs (Lennaerts-Kats et al., 2020). As curative treatments are still unavailable, the economic impact of neurodegenerative diseases is detrimental on societies that are now bearing the cost of long-term palliative care as well as loss of productivity among family members who are forced into caregiver roles (Cantarero-Prieto et al., 2020).

The pathophysiology of synucleinopathies, in brief, involves the misfolding of SNCA proteins in the cytoplasm of neuron cells. The affected cells are either unable or fail to eliminate the misfolded proteins and as the misfolded SNCA proteins aggregate into insoluble amyloids and accumulate within the cells, functional disruptions occur (Melki, 2015; Rodriguez et al., 2018). The regions of the CNS where SNCA aggregates proliferate are specific to each disease and the progressive neurodegenerative decline associated with the respective diseases follow divergent but predictable sequences impacting numerous pathways (Mehra et al., 2019). Such divergent pathologies despite the same originating protein error is possibly explained by the prion strain hypothesis, whereby a single SNCA protein sequence could potentially misfold into the distinct disease-causing amyloid structures (Hoppe et al., 2021). The normal function of SNCA is still the subject of numerous studies (Bendor et al., 2013; Kaur & Lee, 2021; Li et al., 2020; Hernández-Vargas et al., 2021), with most recognising a duality in the role of SNCA as either a physiological neuroprotector of synapses or a pathological inducer of neurodegeneration (Bonini & Giasson, 2005).

The zebrafish is a common laboratory animal model, particularly for developmental biology and neuroscience (Kimmel, 1989; Kimmel, 1993; Stewart et al., 2014). However, the zebrafish does not possess an ortholog to the human *SNCA*. With SNCA playing such a major role in numerous neurodegenerative pathologies, the question arises whether the zebrafish can be applied as a model for synucleinopathies. This review seeks to catalogue studies reported to date on the use of the zebrafish model in studies pertaining to synucleinopathies. A systematic search of the literature using Scholar Google, PubMed/Medline, and Scopus was done in June – September 2021. The following search strategy was used for each of the three bibliographic databases: Title, abstract, keywords, or topic: ("synuclein" OR "synucleins") AND ("zebrafish"); ("synucleinopathy" OR "synucleinopathies") AND ("zebrafish"); ("Lewy body" OR "Lewy bodies") AND ("zebrafish"); ("aggregates" OR "aggregations") AND ("zebrafish"); ("gut brain axis" OR "gut-brain-axis") AND ("zebrafish"). The search was applied for peer-reviewed articles and reviews written in English and published at any time up to 2021.

Synucleins

The synuclein protein was first described in the nuclei and presynaptic nerve terminals of the *Torpedo californica*, commonly known as the Pacific electric ray (Maroteaux et al., 1988). The neuron-specific *Torpedo californica* synuclein consisted of 143 amino acids and its gene was expressed only in the nervous system (Burré et al., 2018). The cDNA clone encoding *T. californica* synuclein was used to isolate a 140-amino-acid synuclein protein in rats. Synuclein homologs were then identified in various vertebrates and three distinct synucleins have been described, namely SNCA, β-synuclein (SNCB) and γ-synuclein (SNCG) (George, 2001). The synucleins are small water-soluble proteins that have a highly conserved amino-terminal domain that includes a variable number of 11-residue repeats and a less-conserved carboxy-terminal domain (George, 2001). Each synuclein is between 127 to 140 amino acids in length, sharing 55% to 62% sequence similarity (Goedert & Spillantini, 2012). The protein homology between SNCA and SNCB have a similarity of 62% and are co-localised within presynaptic nerve terminals in the CNS, whereas SNCG is primarily expressed in the peripheral nervous system (Goedert, 2001; George, 2001; Jakes et al., 1994).

Human *SNCA* is located on chromosome 4q21, encoding 140 amino acids and producing a 14 kDa protein (Bendor et al., 2013; Lee & Trojanowski, 2006). The SNCA amino acid sequence is divided into 3 regions with each region possessing different functions and characteristics (Beyer, 2006). The highly conserved N-terminal domain acquires an α-helical structure

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through binding to phospholipids which suggests SNCA could have a role in lipid membrane binding (Leong et al., 2009). The central hydrophobic non-beta amyloid component (NAC) region is responsible for the aggregation process which is essential for SNCA toxicity. The third, a strong negative charged acidic carboxyl-terminal domain is important for the chaperone-like activity of SNCA, which could aid in proper folding of proteins and inhibit irreversible aggregation of proteins (Periquet et al., 2007; Recchia et al., 2004). In normal conditions, SNCA is expressed abundantly in the presynaptic nerve terminals (Bendor et al., 2013; Stefanis, 2012) of the brain striatum, substantia nigra, hippocampus, neocortex, cerebellum and thalamus (Ahmad et al., 2007). The normal function of SNCA has been reported to be as an essential presynaptic, activity-dependent negative regulator of dopamine neurotransmission (Abeliovich et al., 2000). However, the absence of SNCA does not appear to be detrimental, as observed in an inbred mouse strain C57BL/6S that had a spontaneous deletion of the *SNCA* locus (Specht & Schoepfer, 2001).

Under pathological conditions, the aggregated form of SNCA is the main constituent of LBs and Lewy neurites (Spillantini et al., 1997). It is still up for debate whether the increased expression of SNCA is causative in nature or a defence mechanism towards encroaching disease. It remains unclear whether the abnormally aggregated SNCA protein confers neuroprotection or directs neurotoxicity towards neuronal cells (Luo et al., 2007). In PD pathogenesis, SNCA has been implicated in ubiquitination (Anderson et al., 2006), phospholipid binding (Jo et al., 2000), tyrosine hydroxylase regulation (Perez et al., 2002), and chaperone function (Ostrerova et al., 1999). Point mutations of the *SNCA* gene have been discovered (Appel-Cresswell et al., 2013; Conway et al., 1998; Krüger et al., 1998; Lesage et al., 2013; Polymeropoulos et al., 1997; Proukakis et al., 2013; Zarranz et al., 2004) that lead to early onset forms of α-synucleinopathy (Houlden & Singleton, 2012). Two of these mutations promote the increased formation of large protofibrils which are intermediate aggregates, and induce membrane permeabilization via lipid membrane binding which in turn increases neurotoxicity (Conway et al., 1998; Nuytemans et al., 2010). As reviewed by Ingelsson (Ingelsson, 2016), SNCA's neurotoxicity can compromise cell membrane integrity, synaptic toxicity, impairment in intracellular degradation, mitochondrial toxicity, dysfunction of the endoplasmic reticulum, inflammatory response, and cell-to-cell propagation.

Alternatively, SNCA expression correlates with neuroprotection. *In vitro* studies showed that neurons expressing SNCA after oxidative stress induction were more resistant to apoptotic changes compared to non-expressing neurons (Quilty et al., 2006). This neuroprotective effect was further supported by Chong et al. (Choong & Say, 2011), who reported that wild type SNCA was able to rescue chronic rotenone-exposed SH-SY5Y cells from acute hydrogen peroxide insult. It was also suggested that the increase in SNCA released from neurons could trigger astrocytes to provide a neuroprotective environment (Lee et al., 2010). Further studies supported a 'two hit' hypothesis whereby mild stress to the neurons would initiate a protective up-regulation of SNCA, while an increased stressed environment would intensify SNCA accumulation together with ubiquitin which is central to proteosome-mediated protein degeneration (Musgrove et al., 2011).

Human SNCB consists of a 134 amino acid protein encoded by the *SNCB* gene on chromosome 5q35 (Beyer et al., 2011). While SNCB and SNCA have similar expression patterns and localization in the brain (George, 2001), the structural difference between the two synucleins is that SNCB lacks the central NAC region compared to SNCA (Ahmad et al., 2007). Where SNCA has been implicated in neurotoxicity, SNCB has been proposed to play a neuroprotective role in impeding further aggregation of SNCA protofibrils (Hashimoto et al., 2001; Park & Lansbury, 2003) via its non-amyloidogenic properties (Ahmad et al., 2007). Increased SNCB protein levels have also been reported to reduce SNCA protein aggregation and expression, improving motor performance and survival rates of SNCB transgenic mice (Fan et al., 2006).

Lastly, human *SNCG*, also known as the breast cancer-specific gene 1 (*BCSG1*) is located at chromosome 10q23, encoding 127 amino acids (Lavedan et al., 1998). The SNCG protein (SNCG) is mainly expressed at the axons and cell bodies of primary sensory neurons, peripheral nervous system motor neurons and sympathetic neurons (Ahmad et al., 2007). In the brain, SNCG plays a role in dopamine neurotransmission (Senior et al., 2008). In amyotrophic lateral sclerosis patients, SNCG aggregation has been implicated in the pathogenesis of disease (Peters et al., 2015). Overexpression of SNCG has also been observed in a wide range of cancers from advanced infiltrating breast carcinoma, ovarian tumours and cervical cancer to liver and lung cancers which suggest its association with malignancy, tumour progression and metastasis (Ahmad et al., 2007; Lavedan et al., 1998; Zou et al., 2012).

Zebrafish

The zebrafish (*Danio rerio*) is a teleost fish from the minnow family originating from South Asia which has become established as a laboratory animal in addition to being a common household pet for aquarists (Aleström et al., 2020; Engeszer et al., 2007; Varga, 2018). Teleosts are ray-finned fish that make up 96% of all fishes and are believed to have emerged 260 million years ago. As teleosts are vertebrates, this makes them a more relatable model for human conditions as compared to other popular non-mammalian models, namely *Drosophila melanogaster* and *Caenorhabditis elegans*. Indeed, fishes are the oldest and the most diverse class of vertebrates and all extant vertebrates are said to be descendants of an ancestral fish (Elgar, 2004; Volff, 2005). Fish underwent large-scale gene expansion early in their evolutionary history (Ravi & Venkatesh, 2018). Two rounds of whole genome duplications resulted in four times as many genes in vertebrates as there are in invertebrates (Dehal & Boore, 2005), thus establishing the incredible gene diversity that vertebrates possess. Over time, many of these genes disappeared but there were also many that were retained in teleosts. The teleosts, especially zebrafish, then experienced more recent lineage-specific duplication events that gave rise to species-specific duplicate genes (Lu et al., 2012).

The zebrafish genome has been fully sequenced, revealing zebrafish as possessing 26,206 protein-coding genes; for comparison, humans are reported as having 19,116 protein-coding genes (Howe et al., 2013; Piovesan et al., 2019). Of the human genes, 71.4% have at least one zebrafish ortholog, of which 47% of such human genes have a one-to-one relationship with a zebrafish ortholog. For the human genes that do not have matching zebrafish orthologs, the functions encoded by such genes may instead be performed by functionally similar zebrafish proteins (Barbazuk et al., 2000; Howe et al., 2013). Genetic sequences may have diverged significantly between species (Howe, 2020; Siddiqui et al., 2016) but conservation of functional properties can be uncovered. Hence, while the *SNCA* gene itself is absent in the zebrafish genome, its function very likely is conserved in the zebrafish. Zebrafish are already being used as animal models for neurodegenerative diseases that include synucleinopathies such as PD (Makhija & Jagtap, 2014; Najib et al., 2020; Wang et al., 2017) and taupathies in Alzheimer's Disease (Bai & Burton, 2011; Ding et al., 2019). In the case of the latter, zebrafish have been shown to possess the *mapta* and *maptb* duplicate orthologs of the microtubule-associated protein tau (*MAPT*) implicated in the formation of neurofibrillary tangles in the brains of patients with Alzheimer's disease (Chen et al., 2009a) and observable tau changes were reported in zebrafish adults exposed to hypoxia to mimic stroke or cerebral ischaemia in human patients (Moussavi Nik et al., 2014).

The zebrafish laboratory animal model has many advantages compared to the more common vertebrate rodent and nonhuman primate models. Zebrafish adults are small in size, measuring 4–5 cm in length, and can be housed in aquaculture systems designed to hold hundreds or even thousands of animals within controlled parameters in a single research facility (Aleström et al., 2020). Zebrafish achieve sexual maturity by 3 months of age, breed all year without seasonal variation, and are highly fecund, with a fertile pair producing hundreds of embryos in a week that can be raised and accurately staged by age of development in Petri dishes (Kimmel et al., 1995; Parichy et al., 2009). The embryos develop rapidly and are transparent, allowing for easy stereomicroscopic observation of their internal organs and systems during development, and easily visualised for *in vivo* live analyses with reporter transgenes or immunolabelled *in situ* with RNA probes or antibodies (Godoy et al., 2020; Kuil et al., 2021; Kumar et al., 2019). In addition, the Principles of the 3Rs (Tannenbaum & Bennett, 2015) are important considerations to encourage the use of zebrafish as an alternative vertebrate model that relatively replace the use of mammalian models (Madden et al., 2012). The matter of refinement is also crucial, as in recent years it has become recognised that zebrafish are capable of pain perception and discomfort (Ohnesorge et al., 2021). Just as one would when using mammalian models, suitable analgesia and anaesthesia protocols should be approved by the institution's ethics of animal care and use committee if experiments utilise adult zebrafish (Martins et al., 2016) while early embryo studies can proceed without regulation prior to their free-feeding stage of life (Strähle et al., 2012).

The zebrafish is a highly tractable organism and experiments done on zebrafish can be as simple as exposing the animal to compounds that are dissolved in its water column or by delivering the compound materials into the fish's body via injections or gavage; or can involve forward or reverse genetics via mutagenesis studies, transient knockdowns, knockouts (KOs), and transgenesis (Kalueff et al., 2014; Stewart et al., 2014; Wyatt et al., 2015). In addition to genetic and functional homology, zebrafish are homologous for human cells and organs with the exception of the lungs, and exhibit behaviours that can be monitored and correlated with human disorders (Flinn et al., 2008).

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The zebrafish nervous system is simple but well characterised, and most importantly, it is a vertebrate nervous system (Blader & Strähle, 2000). All life stages of zebrafish can be used to model various human pathologies, including neuronal conditions in order to delineate the mechanisms of disease and to discover new therapies (Barnhill et al., 2020; Choi et al., 2021; Fontana et al., 2018; Keller & Keller, 2018; Maximino et al., 2010; Stewart et al., 2014; Thawkar & Kaur, 2021).

Zebrafish synucleins

The *SNCA* is highly conserved gene. A check on the NCBI page of *SNCA* orthologs displays 265 species (NLM, 2016). Notably, one vertebrate missing from the list is *Danio rerio*, the zebrafish. Zebrafish do possess synuclein genes, namely *sncb* and two *γ*-synuclein paralogs, *γ*-synuclein A (*sncga*) and *γ*-synuclein B (*sncgb*), but the absence of *SNCA* is intriguing as the gene is present in other teleosts (Toni & Cioni, 2015). Table 1 shows the similarity between the zebrafish synucleins and SNCAs of several other species. Blastn algorithm was performed to compare somewhat similar sequences between zebrafish *sncb* and *sncga* query sequences aligned the subject sequences of other species' *SNCA* genes. Mostly dissimilar sequences (discontiguous megablast) was used to compare zebrafish *sncgb* query sequences with the other species' *SNCA* subject sequences. Protein-protein BLAST (blastp) was used for all zebrafish synuclein amino acid query sequences aligned with subject sequences of the other species' SNCA proteins.

When first identified, zebrafish Sncb was described as being 70% identical and 82% similar with human SNCB (Sun & Gitler, 2008). Another report placed the similarities for zSyn-*β*, zSyn-*γ*1, and zSyn-*γ*2 proteins at 69%, 47%, and 50% identity to human SNCB and SNCG, respectively; these three synuclein genes were then named *sncb*, *sncga* and *sncgb* according to standard nomenclature conventions (Chen et al., 2009b). Whole-mount *in situ* hybridization (WISH) with RNA probes showed *sncb* is the earliest expressed of the three zebrafish synuclein genes. From the 8-somite to 16-somite stage, *sncb* was detected only in the trigeminal placode. *sncb* expression then expanded to the ventral diencephalon, olfactory placode, ventral tegmentum, and spinal cord neurons before eventually becoming restricted to the brain and retina by 45 hours post fertilisation (hpf) (Sun & Gitler, 2008).

Meanwhile, synteny inference suggested that *sncga* and *sncgb* were paralogs derived from duplication of the same an-

Table 1. BLAST (Altschul et al., 1990) of nucleotide mRNA and amino acid percent identity between zebrafish synucleins and α-synucleins across selected species: human, nonhuman primate, rodents, and a teleost fish

Zebrafish *Danio rerio*	Human *Homo sapiens*	Rhesus monkey *Macaca mulatta*	Rat *Rattus norvegicus*	Mouse *Mus musculus*	Common carp *Cyprinus carpio*
	SNCA NM_000345	Predicted *SNCA* transcript variant X1 XM_015138783	*Snca* NM_019169	*Snca* NM_001042451	*SNCA* GQ169720.1
sncb NM_200969	67.14	67.57	66.97	66.97	65.17
sncga NM_001017567	74.31	74.31	76.39	75.69	74.42
sncgb NM_001020652	70.79	70.79	72.07	72.07	71.08
	SNCA NP_000336	SNCA isoform 1 XP_014994269	Snca NP_062042	Snca NP_001035916	SNCA ACS68572.1
scnb NP_957263	54.05	53.38	54.73	54.73	51.54
sncga NP_001017567	58.33	57.29	58.33	58.33	59.34
sncgb NP_001018488	63.54	62.50	58.77	58.77	63.04
References	Linnertz et al., 2009; Spillantini et al., 1995	Chu & Kordower, 2007; Shi et al., 2017	Jiang et al., 2014; Wang et al., 2016	Musgrove et al., 2014; Touchman et al., 2001	Vaccaro et al., 2015

sncb, *β*-synuclein; *sncga*, *γ*-synuclein A; *sncgb*, *γ*-synuclein B; *SNCA*, *α*-synuclein.

cestral locus that diverged to become the human SNCG (Milanese et al., 2012). *sncga* expression was not detected during early embryogenesis but from 26 hpf onwards, *sncga* could be detected in the spinal cord and hindbrain neurons. By 3 days post fertilisation (dpf) *sncga* expression was restricted to the brain and retina. As for *sncgb*, it was seen to be expressed within the notochord early in embryogenesis but not elsewhere. After 2 dpf, *sncgb* transcripts were not detectable by WISH (Sun & Gitler, 2008) and in temporal expression RT-PCR profiles, the intensity of *sncgb* bands were weaker compared to the other synucleins. In adult tissues, moderate *sncgb* mRNA expression was detected in the testis, kidney and brain, whereas high levels of both *sncb* and *sncga* were seen most abundantly in the brain followed by the eyes (Chen et al., 2009b). Indeed, all three zebrafish synucleins were detected in the adult brain, wherein *sncga* had the strongest expression and *sncgb* the weakest. *sncb* and *sncga* were distributed throughout the gray matter of the brain and spinal cord, with *sncb* being more prominent rostrally and *sncga* more prominently expressed caudally. In the brain, both *sncb* and *sncga* were shown to be co-expressed in catecholaminergic neurons (Milanese et al., 2012) which comprise the dopaminergic system and noradrenergic system. It is the dopaminergic system that is so severely compromised in PD (Braak & Braak, 2000; Dauer & Przedborski, 2003) and it is important to note that zebrafish dopaminergic neurons have been very well mapped and are functionally comparable to the human's (Du et al., 2016; Ma, 2003; Matsui & Sugie, 2017; Xi et al., 2011). A stable transgenic line, Tg (*sncga*:GFP) generated by Tol2-mediated transgenesis (Asakawa & Kawakami, 2009) produced embryos with green fluorescent protein (GFP) signal distribution that recapitulated the spatial expression pattern of *sncga* revealed by WISH (Chen et al., 2009b).

Transient knockdown of a specific gene function during zebrafish development can be achieved by delivering antisense morpholino oligonucleotides (MO) into the fertilised egg. In zebrafish embryo morphants, gene expression begins to recover as the embryos grow and the cellular MO concentration dilutes (Moulton, 2017), allowing for an observation window of gene inhibition for the first 3 to 4 days of embryogenesis (Bedell et al., 2011; Bill et al., 2009; Corey & Abrams, 2001). Having demonstrated that *sncb* and *sncga* were expressed in the dopaminergic neurons, Milanese et al. (2012) targeted the two synucleins for MO knockdown. Inhibition of *sncb* and *sncga* did not result in lethality or systemic defect, and the morphants' CNS appeared normal (Milanese et al., 2012). However, the loss of *sncb* and *sncga* did impair motor function, with the morphants exhibiting obvious hypokinesia between 3 and 4 dpf which resolved to normal spontaneous motor activity by 5 dpf for the single knockdown morphants, while double knockdown morphants took longer to recover. Low dopamine levels were detected in all morphants. Collectively the results suggested *sncb* and *sncga* are not required for CNS morphologic development but are necessary for early development or differentiation of dopamine neurons (Milanese et al., 2012). Very importantly, Milanese et al. (2012) demonstrated the hypokinetic phenotype seen in *sncb* and *sncga* morphants could be rescued by human SNCA mRNA. This would support the idea of functional redundancy between the synuclein genes in zebrafish and explain the loss of SNCA from the zebrafish genome (Sager et al., 2010).

As *sncb* has been identified as the clear ortholog for human *SNCB*, that leaves *sncga* as the presumptive putative functional homolog for *SNCA* in zebrafish. Notably, the zebrafish *sncga* sequence has been reported to contain N-terminal repeats and hydrophobic regions that are similar to *SNCA* (Milanese et al., 2012). In one study, Sncga (referred to as γ-1 by the authors) was firstly shown to form fibrils similar to that seen with human SNCA *in vitro*, and formed intracellular γ1 aggregates *in vivo* when γ-1 was over-expressed (Lulla et al., 2016). The authors subsequently used dithiocarbamate pesticide ziram to induce neurotoxicity and describe synuclein involvement in zebrafish embryos. A previous *in vitro* study had shown ziram to be selectively toxic to dopaminergic neuron cells in primary mesencephalic culture derived from rats, resulting in increased SNCA expression (Chou et al., 2008). In the zebrafish study, γ-1 was shown to be crucial for ziram neurotoxicity as demonstrated by γ-1 MO knockdown embryos being quite protected against ziram-induced damage to telencephalic and diencephalic aminergic neurons, and the use of a molecular tweezer (MT) to disrupt SNCA aggregation being effective against γ-1 as well (Lulla et al., 2016). Taken together, these observations neatly demonstrate the Sncga-like neurotoxic role for zebrafish *sncga*.

The role for SNCB in synucleinopathies is purportedly one of modulation and possible neuroprotection (Fan et al., 2006; Sargent et al., 2018) leading to significant research investment for assessment of therapeutic applications for SNCB (Hashimoto & Spada, 2012; Hashimoto et al., 2001; Park & Lansbury, 2003, Shaltiel-Karyo et al., 2010). In the zebrafish, *sncb* upregulation and sncb aggregation were seen following knockdown of the *leucine-rich repeat kinase 2* (*lrrk2*) gene. In the *lrrk2* morphant embryos, *sncb* aggregated in the diencephalon, midbrain, hind-

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brain, post optic commissure, and very widely in the hindbrain. Zebrafish *lrrk2* morphants also had significant developmental perturbation and neuron loss, including of dopaminergic neurons (Prabhudesai et al., 2016). *LRRK2* mutations are autosomal dominant and are the most common cause of familial PD (Greggio & Cookson, 2009). The interaction between LRRK2 and SNCA and their links to synucleinopathies have been the subject of much research (Gorostidi et al., 2012; O'Hara et al., 2020; Sen & West, 2009; Taymans & Cookson, 2010), and it should be noted that in the Prabhudesai study, SNCB was used as the substitute equivalent to SNCA and was detected by Western blot by using rabbit polyclonal antibody for SNCB (Prabhudesai et al., 2016). Nonetheless, the zebrafish findings were intriguing as SNCB aggregation is not a common report; despite sharing significant sequence similarity with SNCA, SNCB is reportedly resistant to aggregation (Allison et al., 2014). A current search for LRRK2 and SNCB did not unearth any published articles.

Increased *sncb* expression was also reported in zebrafish embryo morphants of phospholipase A2 group VI (*pla2g6*) (Sánchez et al., 2018), the ortholog of *PLA2G6* which has been associated with neurodegenerative diseases (Gregory et al., 2017; Kurian et al., 2008). Sánchez et al. (2018) were interested in the synucleins as links between *PLA2G6* and SNCA have been reported in other animal models; in the absence of SNCA in the zebrafish, *sncb* was picked for CNS analysis in *pla2g6* morphants due to it being expressed in the presynaptic terminals. The increase in *sncb* expression and distribution in the brain in *pla2g6*-deficient embryos was thus correlated with SNCA elevation in PLA2G6 patients and Pla2g6-KO mice, with the implication that presynaptic synuclein aggregation may be a factor in the pathogenesis of PLA2G6 neurodegeneration (Sánchez et al., 2018). A more recent study into the neurotoxicity and teratogenic risks of silica nanoparticles in the aquatic environment assayed *sncb* as one of several neurodevelopment, autophagy and parkinsonism-related genes. The group reported no apparent changes to *sncb* mRNA levels in embryos immersed in silica nanoparticle solutions (Li et al., 2021). From their discussion, it appeared that the group was conflating *sncb* and SNCA; perhaps *sncga* would have been the better gene to assess possible parkinsonism risk in the silica nanoparticle-exposed embryos.

Zebrafish *α*-synuclein (SNCA)?

Interestingly, studies reporting endogenous SNCA in zebrafish have been published. Khotimah et al. (2015a, 2015b) used rotenone to induce neurotoxicity and synuclein aggregation in adult zebrafish (Khotimah et al., 2015a; Khotimah et al., 2015b). Rotenone is a natural compound isolated from the seeds, stems or roots of several plant species and has been commonly used as an insecticide, pesticide and piscicide. Rotenone directly inhibits mitochondrial complex I of the electron transport chain and selectively damages dopaminergic neurons in the substantia nigra, leading to the progressive mitochondrial dysfunction and increased oxidative stress associated with PD, and its correlation with human PD cases is well documented (Johnson & Bobrovskaya, 2015; Tanner et al., 2011). Importantly, rotenone has long been used to induce parkinsonism in animal models (Innos & Hickey, 2021; Lv et al., 2019; Ünal et al., 2019). In the Khotimah et al. (2015a, 2015b) studies, SNCA expression was detected by immunohistochemistry, whereby Western blots showed positive SNCA protein signals of approximately 14–16 kDa in untreated controls while rotenone-induced zebrafish showed high expression of aggregated SNCA proteins of more than 250 kDa (Khotimah et al., 2015a; Khotimah et al., 2015b). Das et al. (2020) also reported SNCA protein detection by Western blot (Das et al., 2020) in a study aimed at assessing the neurodegenerative effects of benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon often contained in industrial effluents (Chepelev et al., 2015; Das et al., 2020). Adult zebrafish exposed to the neurotoxicant exhibited impaired locomotive function evocative of parkinsonism that is associated with dopaminergic neuron or dopamine loss, alongside significant increase in SNCA protein as detected by Western blot (Das et al., 2020). It could be presumed that both these research groups had used commercial antibodies against mammalian SNCA that had sufficient homology to target either zebrafish Sncb or Sncga (Vaz et al., 2018; Yurtsever et al., 2020).

Hu et al. (2017) tested the neurotoxicity risks of titanium dioxide nanoparticles in zebrafish embryos and reported increases in *pink1*, *parkin*, *snca* and *uchl1* expression levels (Hu et al., 2017); these are genes implicated with the formation of LBs and parkinsonism. Titanium dioxide nanoparticle neurotoxicity was not fatal but did cause delayed hatching and morphological deformities. The nanoparticles accumulated in larval brains, resulting in reactive oxygen species (ROS) generation and hypothalamus cell death as well as dopaminergic neuron loss, all which greatly incriminated titanium dioxide nanoparticles as risk factors for PD (Hu et al., 2017). The upregulation of *snca* in response to the nanoparticle neurotoxicity would have been

in line with the observation of parkinsonism *in vivo* if not for the fact that zebrafish do not possess the gene. In the same vein, Zhu et al. (2020) reported PD-like symptoms in zebrafish larvae following exposure to Fenvalerate, a type of pyrethroid used as an insecticide, and evaluated *pink1*, *parkin*, *snca* and *uchl1* transcription by qRT-PCR (Zhu et al., 2020). Fenvalerate exposure led to increased mortality, abnormalities in morphology and locomotor behaviour in surviving larvae, dopaminergic neuron loss and significant upregulation in *pink1*, *parkin* and *uchl1*; but no significant difference in *snca* expression in exposed larvae compared to unexposed controls. Intriguingly, the published primers designed for *snca* (Zhu et al., 2020) were in fact targeting NM_001113636.2, which is the reference sequence for *synuclein, alpha interacting protein (sncaip)* mRNA, an ortholog of human *SNCAIP* which encodes synphilin-1, a neuronal protein that interacts with SNCA and modulates SNCA aggregation (Engelender et al., 1999; Engelender et al., 2000). Synphilin-1 has also been shown to be one of the protein components in LBs (Wakabayashi et al., 2007) and synphilin-1 mutations have been described in sporadic PD (Marx et al., 2003). Studies are ongoing to understand the possibly cytoprotective role of synphilin-1 in PD pathogenesis (Liu et al., 2016; Shishido et al., 2019; Smith et al., 2010; Tanaka et al., 2004; Hernández-Vargas et al., 2011) and the inadvertent reporting of zebrafish *sncaip/synphilin-1* and its seeming non-response to a neurotoxin (Zhu et al., 2020) offers interesting prospects for further investigations into what role synphilin-1 has in the absence of SNCA.

Meanwhile, Keatinge et al. (2015) utilised the zebrafish model precisely because it lacks SNCA. *Glucocerebrosidase 1* (*GBA1*) mutations are the cause of Gaucher's Disease, an autosomal recessive lysosomal disorder (Hruska et al., 2008). Keatinge's group was focused on understanding the mechanisms of *GBA1* mutation in contributing to increased risk of PD in heterozygous *GBA1*$^{+/-}$ carriers, independent of SNCA toxicity (Keatinge et al., 2015). A *gba1*$^{-/-}$ zebrafish line was generated, and juvenile homozygous and heterozygous mutants of the line showed phenotypes associated with Gaucher's Disease but *gba1*$^{-/-}$ zebrafish did not survive past 14 weeks post fertilization (wpf). Without any SNCA influence, dopaminergic neuron degeneration was clearly detected in *gba1*$^{-/-}$ mutants by 12 wpf. Notably both Sncb and Sncga protein levels were markedly reduced in the *gba1*$^{-/-}$ brains, likely as a consequence of the loss of neuron cells. This study introduced a niche application for the zebrafish in further studies of PD-related pathophysiology unrelated to α-synucleinopathy.

Humanized zebrafish

The high level of genomic conservation between humans and zebrafish allows for the expression of human genes in zebrafish (Barbereau et al., 2021; Cornet et al., 2018, Slijkerman et al., 2018). Of specific relevance to this review, transgenesis of human *SNCA* in zebrafish benefits from of the absence of endogenous SNCA wherein findings would be reflective of the human SNCA rather than complicated by an endogenous protein ortholog, as is seen in transgenic SNCA mammalian models (Visanji et al., 2016).

The first published synucleinopathy study using a humanized zebrafish model was by Prabhudesai et al. (2012) who generated a transgenic zebrafish model of SNCA toxicity (α-syn-ZF) exogenously expressing *SNCA* fused with DsRed as the reporter gene with HuC neuronal promoter and a T2A peptide inserted between the *SNCA* and DsRed sequence (HuC-α-syn-T2A-DsRed). The T2A peptide would be cleaved post-translationally to release SNCA and DsRed in neurons. Embryos injected with the HuC-α-syn-T2A-DsRed construct exhibited DsRed fluorescence in neurons beginning from 12 hpf but also suffered severe morphologic deformities and most were dead by 240 hpf. Severe phenotype and lethality were also seen in embryos injected with HuC-α-syn construct without T2A and DsRed, but not in control embryos expressing HuC-T2A-DsRed, thus implicating SNCA as the cause of toxicity. Additionally, SNCA aggregates were detected in the neurons of α-syn-ZF larvae. However, the SNCA aggregation was prevented when the α-syn-ZF larvae were treated with a novel MT capable of inhibiting the assembly and toxicity of amyloidogenic proteins, thus providing validation for the MT as a potential treatment option for synucleinopathy (Prabhudesai et al., 2012).

O'Donnell et al. (2014) injected their αSyn-2A-GFP transgene construct into single-cell embryos to overexpress human SNCA in the Rohon-Beard neurons, which are peripheral mechanosensory neurons located in the dorsal spinal cord of fish and amphibians. Use of a viral 2A system (Luke & Ryan, 2018; Provost et al., 2007) offered a brighter reporter expression than the earlier α-syn-ZF transgenic (Prabhudesai et al., 2012). Morphology and survival of this GFP α-syn transgenic was also improved compared to the α-syn-ZF model, at least when the *CREST3* sensory neuron (Uemura et al., 2005) enhancer rather than *HuC* promoter was used to drive the GFP *SNCA* expression. Here, SNCA aggregation and axon degeneration followed by cell death were seen in the neurons of embryos expressing human SNCA and GFP, but not in controls expressing GFP

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alone (O'Donnell et al., 2014). O'Donnell et al. (2014) were observing the pathological effects of accumulated SNCA on axons (Tagliaferro & Burke, 2016) as well as on the rate of Wallerian degeneration (Conforti et al., 2014; Rotshenker, 2011) via time-lapse confocal microscopy imaging after axonal injury and determined that Wallerian degeneration occurred in the same manner in SNCA-expressing axons and wildtype axons. The axon-protective protein Wallerian degeneration slow (Wld^S) (Lingor et al., 2012) was shown to afford some protection against SNCA toxicity but could not prevent neuron cell death. However, the transcriptional coactivator PGC-1α (Northam & LeMoine, 2019) was able to protect against both neuron demise and axonopathy (O'Donnell et al., 2014), further validating PGC-1α as a therapeutic target in treating synucleinopathies (Mudò et al., 2012; Ng et al., 2017; Yang et al., 2020; Zheng et al., 2010) and establishing this model for other therapeutic target screens. Matsui & Matsui (2017) also generated a GFP reporter human *SNCA* transgenic line but noted that the *SNCA* expression was weak and the fish did not exhibit any SNCA inclusion bodies even after progressing to the advanced age of 18 months. The authors subsequently delivered injections of human SNCA fibrils into the cerebrospinal fluid (CSF) of adult fish. The result of this xenograft was a significant accumulation of human SNCA and SNCA inclusions in the brains of SNCA transgenics, which were not seen in the brains of wildtype zebrafish that underwent the same procedure (Matsui & Matsui, 2017). Notably, the SNCA in the inclusions distributed throughout the brain had undergone post-translational modification phosphorylation at residue Serine-129 (S129). Phosphorylated SNCA S129 has been implicated in the pathological misfolding of SNCA (Anderson et al., 2006; Oueslati et al., 2013) and earmarked as a biomarker for diagnosis of synucleinopathies, including PD (Cariulo et al., 2019). The main achievement of the CSF injections of SNCA fibrils is this study was the demonstration of prion-like propagation of misfolded SNCA, although the authors acknowledged that the brain inclusions seen might have also included endogenous zebrafish synuclein. Prion-like propagation (Tyson et al., 2016) is currently the focus of much intense study to detail the processes and to strategize potential approaches to limit or even stop the loop of misfolded SNCA aggregating and seeding more and more neuron cells (Goedert et al., 2016; Oueslati et al., 2014; Ugalde et al., 2016). The zebrafish can now be added to the stable of animal models for SNCA propagation studies (Bernis et al., 2015).

More recently, Van Laar et al. (2020) constructed a novel transgenic zebrafish using the mCherry reporter and viral 2A system, *Tg (UAS:hsa.SNCA-2A-nls-mCherry)* that co-expressed human *SNCA* in the ventral diencephalic dopaminergic neurons that are homologous to the mammalian nigrostriatal system. The human wildtype SNCA was expressed at low levels as to avoid neurodegeneration and dopaminergic neuronal loss or neurobehavioral abnormalities in the larvae (Van Laar et al., 2020). The establishment of this transgenic line generated on the transparent Casper ($roy^{-/-}$; $nacre^{-/-}$) background (White et al., 2008) allowed for intravital confocal microscopy (Usmani & Mempel, 2021; van Ham et al., 2014) of the whole CNS in the living zebrafish model which would not have been possible in a rodent model. A second transgenic line Tg (UAS:roGFP2-Orp1) was developed to express a ratiometric hydrogen peroxide (H_2O_2) biosensor (Nietzel et al., 2019; Kostyuk et al., 2020) on a Casper background which was then crossed with the SNCA transgenics to allow for live *in vivo* measurement of cytoplasmic H_2O_2 flux in dopaminergic neurons. H_2O_2 is an endogenous ROS that has been implicated either as a cause of SNCA aggregation or as a consequence of SNCA accumulation (Lehtonen et al., 2019; Xu et al., 2015). Following acute exposures to either MPTP or rotenone, increased cytoplasmic H_2O_2 flux was observed in the dopaminergic neurons of the SNCA transgenics. Additionally, upon exposure to H_2O_2, the transgenics' dopaminergic neurons failed to compensate for redox perturbation, reflecting the ROS impact on mitochondrial dysfunction and cellular dysregulation in PD (Dias et al., 2013; Milanese et al., 2018). These transgenics along with an earlier one generated by the same group (Dukes et al., 2016) offer intriguing modalities for *in vivo* imaging of synucleinopathies and PD-related CNS disruptions in a vertebrate model.

Meanwhile, Weston et al. (2021) reported their transgenic line which transiently expressed human SNCA with a C-terminal GFP tag (*SNCA*-GFP) within motor neurons. Their transgenic larvae allowed for tracking of GFP-tagged human wildtype SNCA and A53T point mutation SNCA (Hoenen et al., 2016; Li et al., 2002) in neurons and presynaptic terminals (Weston et al., 2021). The group utilised fluorescence recovery after photobleaching (FRAP) imaging (Ishikawa-Ankerhold et al., 2012) to measure and quantify SNCA mobility in presynaptic neurons, using this method to differentiate between aggregated proteins with limited mobility and non-aggregated SNCA that diffuse freely. This group established the zebrafish larval nervous system as a viable platform to optically study normal and mutant SNCA function as well as SNCA aggregations, and

even detected nitrated SNCA species within the presynaptic terminals (Benner et al., 2008; Chavarría & Souza, 2013) in addition to demonstrating that zebrafish Polo-like kinase (PLK) (Inglis et al., 2009; Mbefo et al., 2010) could phosphorylate human SNCA at S129 in zebrafish neurons. The entire experiment was an elegant use of the zebrafish model to decipher the mechanisms of human SNCA aggregation and open up avenues for further investigations. Table 2 summarises the zebrafish synuclein models that have been discussed.

α-Synuclein (SNCA) and the gut-brain-axis

In humans, SNCA is present within the enteric nervous system

Table 2. Summary of zebrafish synuclein models that have been published

Synuclein	Methodology	Life stage	Observations	References
Endogenous synuclein				
Tg (*sncga*:GFP)	Tg (*sncga*:GFP)	Embryos	Transgenic GFP expression at 3 dpf in spinal cord, habenula, hindbrain, midbrain, eyes, trigeminal ganglion, vagal ganglion, and posterior lateral line ganglion. Habenula expression is asymmetric, larger GFP domain in left habenula than in right habenula.	Chen et al., 2009b
β-synuclein (*sncb*) *γ-1 synuclein* (*sncga*)	MO knockdown (β MO, γ1 MO, β + γ1 MO)	Embryos, larvae	Slight reduction in *slc6a3* (*dopamine transporter, dat*)-positive neurons in double β + γ1 morphants at 2 dpf. Hypokinesia between 3–5 dpf, reduced dopamine levels at 7 dpf. Hypokinesia rescued by human human α-synuclein.	Milanese et al., 2012
β-synuclein	*lrrk2* MO knockdown	Embryos	β-synuclein aggregation in *lrrk2* morphant brain at 3 dpf.	Prabhudesai et al., 2016
γ-1 synuclein (*sncga*)	*γ-1 synuclein* MO	Embryos	Morphants protected from ziram dithiocarbamate toxicity.	Lulla et al., 2016
γ-1 synuclein (*sncga*)	γ1-synuclein overexpression in neurons via *HuC*-ZFγ1-T2A-DsRed injection at single-cell stage	Embryos	Malformation, reduced survival, intracytoplasmic aggregation in neurons at 2 dpf.	Lulla et al., 2016
α-synuclein (presumably *sncb* or *sncga*)	Rotenone (5 μg/L) immersion for 28 days	Adults	Western Blot detection of α-synuclein aggregates in the brain. Hypokinesia, decreased dopamine levels, increased apoptosis, increased α-synuclein, Caspase-3, Caspase-9 expression and decreased BDNF expression in midbrain.	Khotimah et al., 2015a; Khotimah et al., 2015b
α-synuclein (presumably other synuclein ortholog)	Environmental toxins	Larvae, adults	Increased *α-synuclein* in embryos exposed to titanium dioxide nanoparticles. PD-like symptoms in zebrafish larvae but no increase in *α-synuclein* exposed to Fenvalerate type II pyrethroids. Increased α-synuclein in adults exposed to benzo[a]pyrene.	Hu et al., 2017 Zhu et al., 2020 Das et al., 2020
Exogeneous α-synuclein transgene				
Human *α-syn*, *SNCA* (*HuC-α-syn*-T2A-dsRed) transgenic	*SNCA* fused with DsRed reporter gene with *HuC* neuronal promoter and T2A peptide inserted between *SNCA* and DsRed sequence. Transgene injected into embryos at one-cell stage.	Embryos	*HuC* promoter used to drive *α-syn* DsRed expression in neurons. Embryos have morphological deformities, marked increase in neuron apoptosis as early as 24 hpf, aggregated α-syn in DsRed-positive neurons. Almost all dead by 240 hpf.	Prabhudesai et al., 2012
Human *α-synuclein*, *SNCA* (*aSyn*-2A-GFP) transgenic	*CREST3*:Gal4:UAS:aSyn-2A-GFP transgene injected into wildtype embryos at one-cell stage	Embryos	*CREST3* enhancer used to drive expression in peripheral sensory neurons. Most embryos morphologically normal. Human *α-syn* GFP expression in Rohon-Beard neurons. α-synuclein aggregation by 2 dpf, reduced mitochondrial transport in axons at 2 dpf, axonopathy (axon swelling, beading, fragmentation, degeneration) by 3 dpf.	O'Donnell et al., 2014

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Table 2. Continued

Synuclein	Methodology	Life stage	Observations	References
Human α-synuclein xenograft and human *α-synuclein* (α-syn) transgenic	Human recombinant α-synuclein protein injected into the cerebrospinal fluid (CSF) of 3-month-old AB wildtype and α-syn transgenic (*Xenopus* neural-specific beta tubulin (NBT) promoter:human *α-synuclein* IRES GFP) fish	Adults	α-syn transgenic adults with very weak human α-synuclein expression and absence of α-synuclein inclusion bodies at 18-months-old. At 3 months-post-injection, robust accumulation of human α-synuclein and presence of α-synuclein inclusion bodies throughout the brain in 6-month-old α-syn transgenic fish but not in wildtype fish.	Matsui & Matsui, 2017
Human α-synuclein, *SNCA* (α-Syn) transgenic	Tg (*UAS:hsa.SNCA-2A-nls-mCherry*) zebrafish expressing human wildtype α-synuclein crossed with Tg (*otpb:gal4*) Gal4 driver zebrafish line to activate UAS enchancer	Larvae	Co-expression of human α-synuclein (*mCherry* reporter) in ventral diencephalic dopaminergic neurons at low levels to avoid neurodegeneration or neurobehavioral abnormalities, with fluorescent biosensors for cytoplasmic peroxide flux and glutathione oxidation detection.	Van Laar et al., 2020
Human α-synuclein, *SNCA* (α-synuclein-GFP; A53T α-synuclein-GFP; S129A α-synuclein-GFP; S129D α-synuclein-GFP) transgenics	Human *α-synuclein* tagged with C-terminal Green Fluorescent Protein (α-synuclein-GFP) and zebrafish *neuroD* promoter. Transgene injected into AB/TL wildtype embryos at one-cell stage	Larvae	Human *α-synuclein*-GFP expression appearing at 2–4 dpf in the soma, axon, and presynaptic terminals of central and peripheral nervous system neurons, including some motor neurons projecting from the spinal cord. Robust expression persisting and neurons appeared healthy until at least 8 dpf. α-synuclein-GFP enriched in presynaptic terminals of neurons at 4 dpf for fluorescence recovery after photobleaching (FRAP) imaging detection of freely diffusing, synaptic vesicle-bound, and aggregated α-synuclein; and immunohistochemistry detection of phosphorylated α-synuclein.	Weston et al., 2021

sncga, γ-synuclein A; GFP, green fluorescent protein; dpf, days post fertilisation; MO, morpholino oligonucleotides; lrrk2, leucine-rich repeat kinase 2; BDNF, brain-derived neurotrophic factor; PD, Parkinson's disease; *SNCA*, α-synuclein; hpf, hours post fertilisation; CSF, cerebrospinal fluid; S129, Serine-129.

(ENS) but ENS SNCA levels are significantly higher in PD patients, suggestive of altered enteric SNCA. Additionally, LBs have been detected in the gastrointestinal tracts (GITs) of PD patients (Punsoni et al., 2019). In addition to parkinsonism, another common feature of PD is constipation which often predates the motor and posture dysfunctions (Adams-Carr et al., 2016), thus strongly implicating gut involvement. To explain the pathophysiology of idiopathic PD, Braak et al. (2003) had hypothesized that misfolded SNCA aggregates propagated in the gut and then spread to the CNS via postganglionic enteric neurons and the vagus nerve (Braak et al., 2003). Specifically, Braak's hypothesis put forth that the genesis of idiopatic PD is a yet to be known pathogen or perhaps environmental toxin that is inhaled via the nasal cavity, affecting the olfactory bulb, and is also swallowed, leading to the pathogen being present in the gut and triggering inflammatory reactions that cascade into pathogenic SNCA deposition in the GIT ENS before secondary spreading to the brain (Hawkes et al., 2007). There have been substantial published *in vitro*, *in vivo*, and clinical evidence to give credence to this 'body-first' hypothesis (Rietdijk et al., 2017), but there have also been counterarguments that synucleinopathies are 'brain-first', wherein aggregated SNCA originate in the brain without any peripheral autonomic nervous system involvement (Beach et al., 2021; Borghammer et al., 2021; Lionnet et al., 2018).

Whether or not Braak's hypothesis stands, there is significant focus on the involvement of gut microbiota in *α*-synucleinopathies such as PD (Haikal et al., 2019; Kaur et al., 2021; Liu et al., 2020, Shen et al., 2021). Sampson et al. (2016) demonstrated that parkinsonism motor symptoms would develop by

transplanting gut bacteria from PD patients into the GIT of SNCA-overexpressing mice (Sampson et al., 2016). Several reviews of animals models utilised for investigating SNCA and the gut, highlighted the challenges involved in deciphering and translating findings to the human condition (Rey et al., 2016; Van Den Berge & Ulusoy, 2022; Vascellari et al., 2021). This review will be summarising zebrafish gut-brain-axis research and the applicability for zebrafish to model transneural SNCA propagation.

Zebrafish gut-brain-axis models

Zebrafish ENS development and gut motility have been well described and gene orthologs identified (Baker et al., 2019; Ganz, 2018; Olsson et al., 2008; Shepherd & Eisen, 2011). Zebrafish gut and enteric innervation are functional as early as 5 dpf and are highly conserved if somewhat simpler compared to the mammalian gut and ENS (Kuil et al., 2021), and the mechanisms of ENS regeneration have been described in zebrafish larvae (Ohno et al., 2021). The studies discussed earlier in this review detailed synuclein gene expressions in zebrafish that were focused quite exclusively on the CNS and spinal cord or limited their characterisations to the embryo and larval stages (Chen et al., 2009b; Sun & Gitler, 2008). mRNA expression levels of zebrafish synuclein transcripts in adult tissues were nonetheless shown to be highest in the brain and low or undetectable in the gut (Chen et al., 2009b). This does not however preclude endogenous synuclein presence in the zebrafish ENS. Determination of the expression level of *sncga* in the gut of zebrafish that are treated with rotenone or other parkinsonism-inducing agents could provide for some interesting options in the use of the zebrafish model to chart transneural propagation of synuclein proteins from the gut to the brain or vice-versa.

Zebrafish gut-brain-axis can be studied at larval and adult stages. Zebrafish *sox10* mutant larvae that completely lacked the ENS and as a consequence suffered from bacterial overgrowth, dysbiosis and intestinal hyper-inflammation could be rescued somewhat by transplantation of wildtype ENS precursors into the mutants or by colonizing the *sox10* mutant larval intestine with anti-inflammatory bacteria isolates from a healthy human (Rolig et al., 2017). Borrelli et al. (2016) were the first to report an adult zebrafish gut–brain-axis model. Adult wild type zebrafish between 4–6-months-old were fed probiotics twice daily for 28 days to induce changes in enteric microbiota, neurochemistry and behaviour (Borrelli et al., 2016), thus establishing the feasibility of the model for microbiota and gut-brain crosstalk research. Cuomo et al. (2021) then utilised the model to correlate behavioural changes with DNA methylation epigenetics and the microbiome following probiotic feeding (Cuomo et al., 2021).

As more data about PD patients' gut microbiota become known (Ma et al., 2019; Pietrucci et al., 2020), the specific bacterial isolates and their impact can be reasonably validated in the zebrafish gut–brain-axis model. Davis et al. (2016) derived germ-free (GF) zebrafish larvae based on earlier protocols for generating gnotobiotic zebrafish larvae (Davis et al., 2016; Pham et al., 2008). The absence of microbiota in GF larvae clearly impacted on locomotor activity and behaviour, which were attenuated by probiotic treatment. The GF larvae were exposed to *Lactobacillus plantarum* directly in the water from 4–6 dpf and although the larvae were too young to really eat, the bacteria could enter into and colonise the gut to establish the microbiome (Davis et al., 2016). Zhang et al. (2021) used GF adults in addition to conventional laboratory fish to study gut microbiota regulation of neurotransmitter secretion (Zhang et al., 2021). Shotgun metagenomic sequencing was performed to identify different microbiota groups based on treatment inductions and elucidate a potential mode of action through the microbiota-gut-brain axis for regulation and neuromodulation (Zhang et al., 2021). Similarly designed experiments could be designed using gnotobiotic humanized SNCA fish to understand the impact of microbiota on synucleinopathy.

Gut dysfunction and by extension, microbiota disruption can also be stress-induced to mimic the pathophysiology of neurodegenerative disorders (Dodiya et al., 2020; Johnson et al., 2018). Cansız et al. (2021) exposed adult zebrafish to rotenone for 30 days to induce inflammation and oxidative stress in the gut-brain axis (Cansız et al., 2021). The rotenone-treated fish suffered locomotor defects indicative of CNS dysfunction. The group did not evaluate for synuclein gene expression but did measure pro-inflammation genes and *brain-derived neurotrophic factor* (*bdnf*) in the intestines and brain as they demonstrated that caprylic acid could ameliorate neurotoxin-induced inflammation and oxidative stress and augment locomotor activity (Cansız et al., 2021). Administration of caprylic (octanoic) acid by oral gavage to the rotenone-treated zebrafish was done to mimic ketogenic diets that have been reported to have therapeutic potential for PD (Cheng et al., 2009; Shaafi et al., 2016) and the use of the zebrafish model in this manner could be readily applied to oral drug discoveries for synucleinopathies.

There have been recent reviews that can be referenced on this topic. de Abreu et al. (2019) delineated the core gut microbiome between humans, rodents, and zebrafish, and discussed

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zebrafish gut-brain crosstalk and the effects of zebrafish gut microbiota on different systems and on behaviour (de Abreu et al., 2019). Bertotto et al. (2020) reviewed the zebrafish microbiota and detailed larval bacterial gut colonisation methods and experimental assays (Bertotto et al., 2020) as well as the use of zebrafish for studying xenobiotics (Koppel et al., 2017) while Mohanta et al. detailed the zebrafish microbiota-gut-brain-axis homeostasis and mechanisms of brain functioning and modulation of brain neurochemistry (Mohanta et al., 2020). Most recently, Lee et al. (2021) provided a thorough review on using the zebrafish for investigating the association between microbiota-gut-brain-axis and neurological disorders which can include synucleinopathies, and dived into utilising mutant KOs, knockins, and transgenic reporter zebrafish lines to fully exploit this model (Lee et al., 2021).

Conclusion

The zebrafish is a highly amenable research model for investigating synucleinopathies by either utilising the zebrafish's endogenous synucleins that are compensating for the absence of SNCA or by generating humanized transgenics that express the human SNCA. The different forms of synucleinopathies can be recapitulated in this model, which could shed light on the non-PD disorders that have been somewhat overshadowed by the focus on PD. Advanced *in vivo* imaging techniques can now be applied to visualise everything from unique bacterial species in the larval gut to cellular flux in the brain, providing incredible troves of evidence to sieve through for analysis. The establishment of synucleinopathy models would also lead to the feasibility of performing high-throughput screening assays to identify new therapeutics for targeted therapy against synuclein aggregation or to protect neurons from the aggressive onslaught of SNCAs.

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Availability of data and materials

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