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Caenorhabditis elegans as an emerging high throughput chronotherapeutic drug screening platform for human neurodegenerative disorders☆

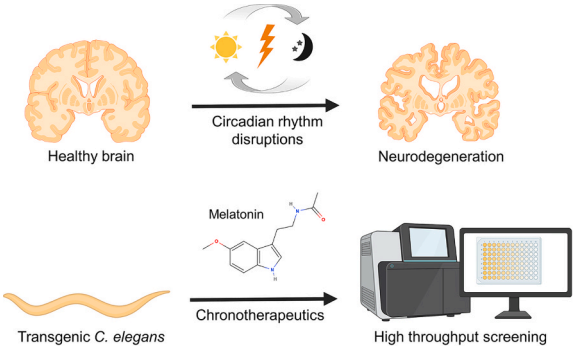
Mrutyunjaya Panda [a,b,c,1], Maria Fakitsa [a,d,1], Maria Markaki [a], Nektarios Tavernarakis [a,d,*]

[a] Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion 70013 Crete, Greece
[b] Department of Medicine, University of Verona, Verona 37134, Italy
[c] Faculty of Pharmacy, University of Lisbon, Lisbon 1649-003, Portugal
[d] Division of Basic Sciences, School of Medicine, University of Crete, Heraklion 71003 Crete, Greece

HIGHLIGHTS

- *C. elegans* is a versatile tool for high throughput chronotherapeutic drug screening against neurodegenerative disorders.
- Animal studies have advanced our understanding of the molecular processes linking circadian rhythms and neurodegeneration.
- Neurodegenerative disorders share mechanistic and pathophysiological features, including circadian rhythms disruptions.
- Animal models are useful platforms for screening chronotherapeutic drug candidates targeting the circadian clock in neurodegeneration.

GRAPHICAL ABSTRACT



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ABSTRACT

An increase in the aging population is accompanied by increased susceptibility to age-associated neurodegeneration, with currently no cure. Despite the diversity of symptoms and etiologies, neurodegenerative disorders share mechanistic commonalities and many pathophysiological features. These include disruptions in circadian rhythms that affect neuronal physiology. Systematic investigations in several animal models have advanced our understanding of the molecular processes that link circadian rhythms and neurodegenerative disease states. These models have also been used to screen and validate promising chronotherapeutic drug candidates that target the circadian clock to ameliorate neurodegeneration. With the emergence of robust and reliable methodologies to measure daily rhythms, the nematode model *Caenorhabditis elegans* has become a versatile tool for high throughput chronotherapeutic drug screening against neurodegenerative disorders. In this review, we discuss the unique features and advantages of *C. elegans* as an enabling platform for chronotherapeutic drug discovery, towards the development of innovative strategies for the treatment of human neurodegenerative conditions.

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* Corresponding author at: Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology-Hellas, Heraklion 70013, Crete, Greece.
E-mail address: tavernarakis@imbb.forth.gr (N. Tavernarakis).

[1] M. Panda and M. Fakitsa are co-first authors.

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1. Introduction

Neurodegenerative disorders (NDs) are a leading cause of disability and death affecting millions of individuals worldwide. As the average lifespan of the population increases, the number of elderly people affected by NDs is also increasing. In the absence of effective treatment, NDs have emerged as a major global health concern [1].

Circadian rhythms play a critical role in maintaining homeostasis at both systemic and tissue levels. From a translational perspective, cyclical changes in transcription have been observed in 82.2 % of the genes coding for proteins which are identified as druggable targets by the U.S. Food and Drug Administration (FDA) [2]. Therefore, rhythmicity has a major regulatory impact on the health of an organism. Disruptions in circadian rhythms lead to physiological imbalances, facilitating the onset and progression of neurodegeneration. Restoring proper timing and coordination between tissues could offer a promising approach for developing new therapeutic interventions.

Chronotherapeutics refers to therapeutic strategies timed to align with endogenous biological rhythms to optimize disease recovery and minimize potential side effects. It considers (a) endogenous oscillations, (b) rhythmic patterns of disease pathology, and (c) optimized chronopharmacological regimens [3]. Chronotherapeutics is emerging as a leading candidate for the management of neurodegeneration. Pharmacological clock modulators such as melatonin, melatonin receptor agonists (e.g., agomelatine, ramelteon, tasimelteon, TIK-301) and small molecules (e.g., lithium, nobiletin and SR9009) have been used extensively to manage several NDs [4–6].

Cell-based and multicellular animal models have been valuable tools for research on neurodegeneration [7]. Given the conservation of genes and neural signalling pathways across vertebrate and invertebrate species, researchers have focused on the nematode *Caenorhabditis elegans* (*C. elegans*) to identify the mechanisms underlying ND pathology. Over the past few decades, *C. elegans* has been extensively used as a model organism in neurobiology and beyond. Advances in genetic engineering, along with a relatively simple nervous system and mapped neuronal networks in worms, have helped immensely in the study of neuronal function and dysfunction. Systematic bioinformatic approaches, which have already been established for cardiovascular research [8], can also be used to elucidate conserved genes and pathways in *C. elegans* models for neurodegeneration.

Multiple human NDs have been modelled in *C. elegans* to investigate disease mechanisms, identify potential drug targets, and screen therapeutic compounds for drug development [9]. Drug screening using *C. elegans* as a model has gained popularity with the rise of high throughput methods [10]. Indeed, recent advances in liquid workflows, along with the automation of imaging platforms and data analysis, have made *C. elegans* a versatile platform for high throughput drug screening approaches (discussed in section 7.2). In this review, we highlight the relevance of the *C. elegans* model as a high throughput chronotherapeutic drug screening platform for discovering new bioactive compounds against neurodegeneration.

2. The nematode *C. elegans*

C. elegans is a small worm that lives in soil and feeds on bacteria. It can be easily cultured in the laboratory using medium supplemented with *Escherichia coli* (usually with the strain OP50). The majority of *C. elegans* are hermaphrodites, enabling the development and maintenance of genetically identical populations. Males exist at a much lower frequency (less than 0.2 %), which allows for genetic cross-processes. An adult hermaphrodite worm consists of 959 somatic cells, whereas an adult male is composed of 1031 cells. *C. elegans* has a short life cycle of about 3 days from eggs to egg-laying adults under optimal conditions. Following the reproductive stage, wild-type hermaphrodites produce approximately 300 progeny via self-fertilization and over 1000 progeny when cross-fertilized, which can survive for 2–3 weeks under laboratory conditions. Due to its small size and transparent body, *C. elegans* is suitable for non-invasive optical monitoring and manipulation methodologies [11].The anatomical arrangement of all somatic cells in the animal, along with their complete cell lineage, is well-established. Its nervous system, which consists of 302 neurons, has a precise description of the neuronal position and connectome [12,13]. Despite its anatomical simplicity, *C. elegans* displays a range of complex behaviours, such as associative learning and memory [14]. These behaviours can be studied using various methodologies. These include *in vivo* imaging with fluorescence microscopy; molecular and genetic tools, such as CRISPR-Cas genome editing [15] for neuronal activity monitoring and manipulation; systematic, genome-wide and high throughput compound screens, and molecular profiling technologies such as transcriptomics and metabolomics [16,17]. Hence, along with its completely sequenced and annotated genome (~97 Mb), which exhibits a high level of conservation with the human genome, *C. elegans* appears to be a valuable model organism for studying numerous biological processes in both physiological and disease contexts [11].

Modelling human diseases in *C. elegans* requires genetic engineering. This can be achieved either by modifying the expression levels of disease-related homolog genes or by overexpressing human isoforms throughout the nematode or in specific tissues. Thus, mutant and transgenic animals displaying phenotypes similar to human pathologies can be developed [18].

Given these advantages, *C. elegans* serves as a valuable model for investigating the molecular mechanisms of neuronal physiology and degeneration, providing insight into the cellular and molecular underpinnings of neurodegenerative disease development and progression.

3. Neurodegenerative disorders

Neurodegeneration is a major public health challenge affecting a large proportion of the population worldwide. Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and Amyotrophic Lateral Sclerosis (ALS) are the most common NDs [19]. Various animal models have been developed to enhance our understanding of molecular pathogenesis and to identify new therapeutic targets [7]. Here, we summarize the *C. elegans* models used to study NDs, highlighting their pathological hallmarks that are also observed in humans, thereby establishing *C. elegans* as a versatile preclinical model (Fig. 1).

3.1. Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent ND accounting for 60–80 % of dementia cases, and it is characterized by memory loss and cognitive impairment. Recent predictions indicate that the prevalence of dementia will triple worldwide by 2050. At the cellular level, the most well-known pathological characteristics are amyloid β-peptide (Aβ) plaques and tau neurofibrillary tangles (NFTs). In addition, mutations in the amyloid precursor protein (APP), presenilin 1/2 (PSEN1/2), and apolipoprotein E (APOE) genes are significant risk factors [20].

Several *C. elegans* strains have been developed over time to mimic the pathophysiology of AD. The first strains were based on the expression of Aβ1–42, the most toxic form of the Aβ peptide, in body wall muscle cells. The absence of endogenous Aβ caused the accumulation of toxic Aβ oligomers and eventually lead to age-associated paralysis [16,21]. Similarly, neurodegeneration was observed in worms that overexpressed Aβ1–42 in glutamatergic neurons [22], whereas pan-neuronal Aβ1–42 expression led to neuromuscular defects [23].

In addition to amyloid plaques, NFTs, which consist of abnormally phosphorylated tau proteins involved in microtubules stabilization, serve as another pathological feature [20]. In *C. elegans*, *ptl-1* encodes a protein homologous to tau, and its absence partially mimics AD pathology [24]. Because of the functional differences between *ptl-1* and tau, *C. elegans* tau models overexpressing human tau have also been developed. Pan-neuronal expression of mutant human tau in *C. elegans*

leads to more severe tau aggregation, impaired locomotion, and overall neuronal dysfunction compared to the expression of wild-type tau [25].

APP plays a crucial role in the production of Aβ peptides. The *C. elegans* ortholog of APP, amyloid precursor-like 1 (*apl-1*) [26], is involved in memory regulation. Its pan-neuronal overexpression impairs both associative and non-associative memory, including olfactory memory and touch habituation [27]. Knockdown of *apl-1* results in faster paralysis in the aldicarb assay, a test used to study neurotransmission, specifically acetylcholine release. As an acetylcholinesterase inhibitor, aldicarb blocks the breakdown of acetylcholine, leading to its accumulation at the synaptic cleft. This accumulation causes overstimulation of postsynaptic receptors, resulting in muscle paralysis. The observed paralysis indicates a defect in synaptic function [28].

Moreover, mutations in PSENs, which encode components of the γ-secretase complex that plays a critical role in processing of Aβ peptides, lead to familial AD [29]. *C. elegans* has three genes that are orthologs to PSENs: *sel-12*, *hop-1*, and *spe-4* [30]. The *spe-4* (spermatogenesis defective 4) gene is expressed only in spermatozoa during spermatogenesis, so it is not relevant to AD, whereas *hop-1* (homolog of presenilin) null mutants have no apparent phenotype. Therefore, *C. elegans* PSEN models are based on *sel-12* mutants, with *sel-12* mutations causing alterations in endoplasmic reticulum and mitochondrial calcium homeostasis, suggesting the need for alternative therapeutic targets in AD [31].

Finally, APOE, which is responsible for cholesterol transport from astrocytes to neurons, is the most prevalent risk factor for sporadic AD [32], with the *APOEε4* allele increasing the risk of developing AD compared to other alleles (*APOEε2* and *APOEε3*) [20]. *C. elegans* does not possess an endogenous ortholog; thus, models expressing human APOE alleles were developed with or without the Aβ1–42 to examine their interactions [33,34]. Pan-neuronal expression of *APOEε4*, but not *APOEε3*, causes neurodegeneration in worms [35], while expressing any of the APOE alleles in glutamatergic neurons in the absence of Aβ, does not lead to neurodegeneration [33]. Hence, more transgenic *C. elegans* models based on APOE are needed to better mimic AD pathology.

3.2. Parkinson's disease

Parkinson's disease (PD) is the second most common ND of the central nervous system, characterized by bradykinesia, tremor and stiffness, affecting approximately one percent of people over the age of 60. At the cellular level, PD is associated with loss of dopaminergic (DA) neurons in the substantia nigra pars compacta, loss of dopamine in the striatum, and the presence of intracytoplasmic inclusions, which are composed mainly of α-synuclein (α-syn) [19]. Like other NDs, most PD cases are sporadic, with 5–10 % of cases showing familial inheritance. To date, at least 19 genes have been linked to familial PD, with the most studied being α-syn and leucine-rich repeat kinase 2 (LRRK2) [36].

α-syn, encoded by the SNCA/PARK1 in humans, is localized at presynaptic terminals and is thought to regulate neurotransmission and synaptic plasticity [37]. Although there are several orthologs of PARK genes in *C. elegans*, they do not include PARK1/SCNA [38]. Thus, *C. elegans* PD models are based on overexpression of human α-syn. The first *C. elegans* α-syn model was developed in 2003, using nematodes overexpressing wild-type and mutant (A53T) forms of α-syn. Their pan-neuronal expression causes age-independent motor defects, but not when expressed only in DA neurons [39]. This may be because, in addition to the dopaminergic pathway, the serotonergic pathway also contributes to *C. elegans* locomotion [40].

Similar to α-syn, mutations in LRRK2 are associated with PD, with the latter being the most common genetic cause of PD [36]. The exact function of LRRK2 is largely unknown. However, it has been linked with oxidative stress, inflammatory responses, mitochondrial and synaptic dysfunction, and the autophagic-lysosomal system [41,42]. *C. elegans* has an ortholog of LRRK2 with a conserved function, *lrk-1*, whose null mutations cause mislocalization of synaptic vesicles [43]. Additionally, there are nematode models that express pan-neuronally the human LRRK2, and in particular its most common mutant form, G2019S [44,45]. These worms are characterized by selective vulnerability of DA neurons [46], which can be rescued by the LRRK2 kinase inhibitors GW5074 and sorafenib [47], suggesting LRRK2 inhibition as a potential intervention for PD.

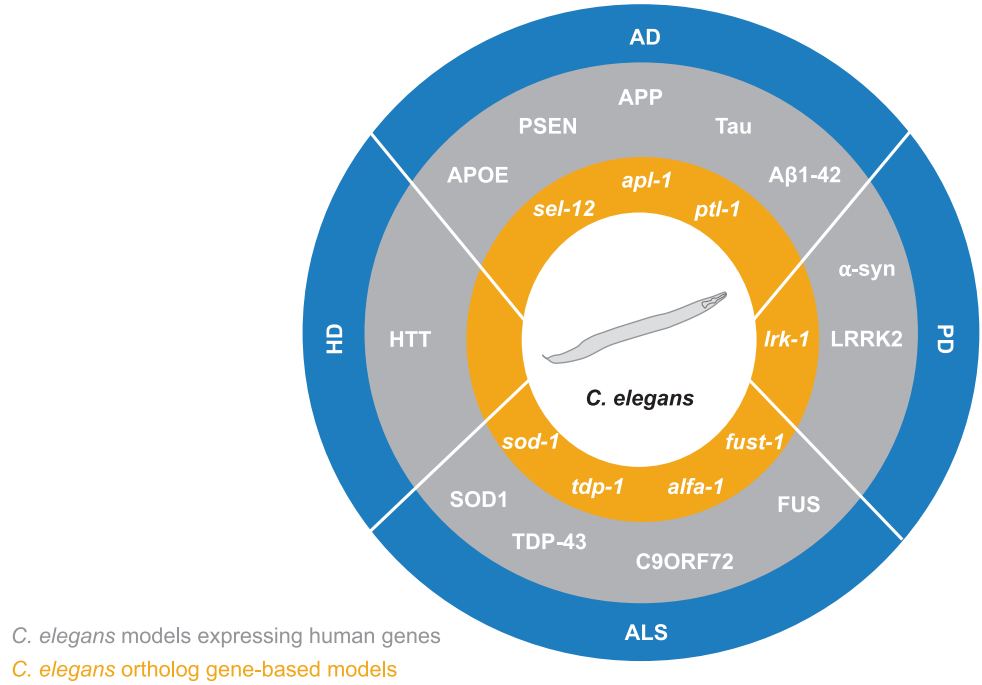

Fig. 1. *C. elegans* as a model system for neurodegenerative disorders. Worms expressing disease-associated genes or the related human gene orthologs.

3.3. Huntington's disease

Huntington's disease (HD) is an autosomal dominant ND caused by CAG trinucleotide repeat expansion in the huntingtin (HTT) gene, which is translated into a long extension of polyglutamine (polyQ) repeats in mutant HTT proteins, leading to neuronal loss. Early age of onset and rapid disease progression are associated with an increased length of CAG repeats, with HD occurring in patients carrying approximately 40 or more CAG repeats. During disease progression, patients experience motor and psychiatric symptoms, including depression, psychosis, and cognitive impairment [48].

C. elegans does not have an HTT ortholog; therefore, the initial models were transgenic animals expressing HTT with different Q lengths in amphid sensory neurons. However, only the expression of the longer HTT with a polyQ track of 150 residues causes age-dependent protein aggregation [49]. These findings are in agreement with the results from mechanosensory neurons expressing HTT with 88 or 128 Q residues, displaying polyQ toxicity with axonal abnormalities and increased protein aggregation [50]. In addition to worms expressing neuronal polyQ, several models have been generated to express different lengths of polyQ in the body wall muscle. These transgenic nematodes exhibit a polyQ repeat length-dependent decrease in body movement [51]. Hence, *C. elegans* is considered a prominent model organism for studying polyglutamine-based diseases, such as HD.

3.4. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron (MN) disorder, characterized by progressive loss of cortical and spinal MNs, leading to muscle weakness and paralysis [19]. Cu/Zn superoxide dismutase 1 (SOD1) was the first gene to be associated with ALS, while chromosome 9 open reading frame 72 (C9orf72), TAR-DNA-binding protein 43 (TDP-43), and fused in sarcoma (FUS) are novel ALS-related genes [52].

Superoxide dismutase 1 (SOD1) catalyses the conversion of superoxide anions to hydrogen peroxide. *C. elegans* has five distinct genes that code for SODs (*sod-1*, *sod-2*, *sod-3*, *sod-4* and *sod-5*); however, SOD1 toxicity in the context of ALS has predominantly been studied in transgenic worms expressing human disease-associated gene [53]. Pan-neuronal or muscle-specific expression of the mutant variant G85R-SOD1 leads to ALS-like phenotype [54]. Recently, the first single-copy/knock-in model for five different SOD1 variants introduced into the *C. elegans sod-1* gene was reported [55]. Interestingly, it was shown that both gain- and loss-of-function mutations in *sod-1* contribute differentially to ALS pathogenesis in distinct neuronal populations, suggesting that the mechanisms underlying neuronal degeneration may vary across different types of neurons.

Additionally, the GGGGCC hexanucleotide repeat expansion (HRE) in the C9orf72 gene, which is the most frequent mutation in familial ALS, is related to impaired nucleocytoplasmic transport and autolysosome pathway, among others [53]. *C. elegans* has an ortholog gene of C9orf72, *alfa-1*, whose deletion mutation leads to age-related paralysis and GABAergic motor neuron degeneration [56]. Recent work has also shown that the *alfa-1* deletion mutant contributes to dysregulation of lysosomal homeostasis, with the expression of human C9orf72 partially rescuing this mutant phenotype [57]. However, transgenic nematodes are more often used to study C9orf72 toxicity because of the lack of a typical HRE in *alfa-1* [58].

Despite the diversity in disease etiologies, 97 % of ALS cases are characterized by TDP-43 positive inclusions [53]. Under physiological conditions, TDP-43 is predominantly localized in the nucleus, where it regulates transcription and alternative splicing. However, in diseased states, it is mislocalized to the cytoplasm, where it is found in inclusion bodies [59]. Depletion of *tdp-1*, the *C. elegans* ortholog, leads to increased stress sensitivity and defects in locomotion, fertility, and growth [60,61]. Additionally, transgenic worm models have been developed to mimic key features of the TDP-43 ALS-like phenotype [62].

Similar to mutations in TDP-43, mutations in the FUS protein cause its mislocalization, leading to the formation of cytoplasmic inclusions that contribute to ND [53]. The ortholog gene in *C. elegans*, *fust-1*, is involved in maintaining the structure and function of neuromuscular junctions and neuronal integrity, and also plays a critical role in regulating lifespan and cellular stress responses [63,64]. Transgenic *C. elegans* models are often used, with ALS-associated FUS leading to impaired communication between motor neurons and muscles [65]. These findings demonstrate that *C. elegans* is an exceptional model for elucidating the molecular mechanisms underlying ALS pathogenesis.

Overall, the usage of *C. elegans* to study NDs offers valuable insights due to its simple nervous system and the well-established genetic tools [66]. Given the conservation of many key pathways involved in neurodegeneration between *C. elegans* and mammals, these findings could pave the way for future studies in mammalian systems. Although *C. elegans* does not fully recapitulate the complex pathophysiology of humans, it allows us to study disease-associated mechanisms at the molecular and cellular levels. Thus, nematode-based studies provide valuable insight into pathological mechanisms and support the model's role in translational research for human NDs.

4. Circadian rhythms

Circadian rhythms are timekeeping cyclic variations in many biological processes or activities. They are entrainable, self-sustaining, and exhibit oscillatory properties to orchestrate molecular, physiological, and behavioural processes. These rhythms are generated, sustained, and synchronized by a hierarchical network of central and peripheral circadian clocks or pacemakers, which operate on an approximately 24-hour periodicity [67].

Zeitgebers are defined as stimuli that regulate endogenous clocks, enabling organisms to adapt to, anticipate, respond to variations, and maintain homeostatic periodicity. These stimuli can be external such as photic cues (i.e., light) or non-photic cues (food availability, temperature, exercise, and social interactions). In addition, hormones like melatonin and cortisol can act as internal zeitgebers, even though they do not function as external cues like light or temperature [68–70]. Diverse physiological parameters and processes, such as feeding, sleep-wake cycles, core body temperature, hepatic metabolism, and renal function, exhibit circadian rhythmicity. These biological rhythms are also observed in numerous biochemical processes and endocrine secretions (cortisol, growth hormone, melatonin, and prolactin) [71,72].

The circadian system can be systematically distinguished by four key elements: (a) a central biochemical master clock with an approximately 24-h time period; (b) input pathways that allow the central clock to synchronize with zeitgebers; (c) various output mechanisms, including endocrine secretions and autonomic innervations, synchronized through specific phases of the central clock; and (d) peripheral molecular clocks present in all cells that control rhythms in global transcriptomic expression, thereby regulating physiology in a tissue-specific manner [67].

The central circadian master clock resides in two paired nuclei in the hypothalamus known as the suprachiasmatic nuclei (SCN), each of which contains approximately 10,000 neurons [73]. Photosensitive ganglionic cells in the retina act as input pathways to transmit photic signals via the retinohypothalamic tract to the SCN [74,75]. Other input pathways from different brain regions and external stimuli are also received by SCN, which collectively entrain and synchronize the circadian clock [76]. This leads to various output mechanisms from the SCN directed to diverse brain regions that regulate endocrine secretions and generate autonomic innervations conveyed to the rest of the body [77].

Molecular regulation of the central clock involves auto-regulatory transcriptional-translational feedback loops (TTFLs) with an approximate 24-h time period [76,78]. During the early light period, the transcription factors CLOCK and BMAL1, encoded by the *clock* and *bmal1*

genes respectively, heterodimerize and translocate to the nucleus to bind to specific DNA E-box enhancer elements stimulating transcription of the target genes Cryptochrome (*Cry1, Cry2*) and Period (*Per1, Per2, Per3*). The CRY and PER proteins encoded by these genes accumulate in the cell over time to form heterotypic complexes which then translocate to the nucleus and repress the transcriptional activity of the CLOCK: BMAL1 heterodimer. This ultimately results in decreased levels of the PER and CRY proteins. During the dark period, heterotypic PER:CRY complexes degrade to very low levels, resulting in the initiation of a new cycle with the transcription of CLOCK and BMAL1. CLOCK:BMAL1 heterodimers initiate the transcription of another feedback loop that involves genes encoding the orphan receptors, RORs and REV-ERBα/β. RORs upregulate while REVERB downregulates the *Bmal1* transcription by competing for the ROR binding element present within the promoter region of *Bmal1*. Additional regulation of the autoregulatory loop is highlighted through a gradient increase in phosphorylated PER, obtained by balancing the levels of CK1ε/δ, which phosphorylates PER for proteasomal degradation, and PP1, which dephosphorylates PER [78–80]. Furthermore, a distinct set of D-box-containing clock output genes under core circulatory loop regulation also exists [81]. These circadian feedback loops and output factors regulate the transcript levels of several genes that feature specific regulatory motifs in their promoter regions, acting as dynamic cellular oscillators [79].

Transcriptomic studies have suggested circadian regulation to be highly tissue-specific [82]. The transcriptional activities of diverse cellular pathways and tissue-specific gene expression are dynamically coordinated by core circadian clock components in each cell [83]. It therefore appears that the variability of rhythmic expression in tissues has evolved to optimize tissue-specific function during the circadian cycle.

4.1. Models to study circadian rhythms

Circadian clocks present an evolutionary advantage for organisms as they coordinate physiological and behavioural processes to maintain homeostatic periodicity in response to changing external conditions. The molecular mechanisms and processes of circadian rhythms share many interspecific similarities, including the TTFL. Certain levels of conservation can also be observed between bacteria and eukaryotes [84]. Canonical clock genes and proteins and their expression are conserved across the animal kingdom [85].

Model organisms have enabled the application of genetic tools and molecular techniques to decipher complex behavioural and physiological processes, such as circadian rhythms. The genetic makeup of circadian rhythms was primarily reported in experiments using *Drosophila* and rodents, where TTFLs are composed of the basic helix–loop–helix/Per-ARNT-SIM (bHLH-PAS) transcription factors acting as core feedback loop regulators. Core clock components are highly conserved among species. However, duplication events leading to non-uniform retention of clock genes have occurred, resulting in diverse functional variations [86].

Historically, *Drosophila melanogaster*, owing to its extraordinary homology with mammalian circadian clock components, has been a suitable model organism for studying the molecular basis of circadian rhythms. However, other animal models are also being explored and adopted, considering their unique experimental advantages [87–89].

As previously mentioned, the nematode *C. elegans* is an effective model for studying complex physiological processes due to its simple anatomy, tractable genome and well-characterized biology. Biological rhythms were initially characterized in *C. elegans* based on the endogenous, temperature-compensated ultradian defecation clock which repeats the defecation motor program every 45 s [90]. The initial descriptions of circadian rhythms in *C. elegans* were observed in the locomotion behaviour or responses to osmotic stress in larvae [91,92]. Other circadian rhythms observed in *C. elegans* include abiotic and biotic stress tolerance [93–95], food and oxygen consumption, pharyngeal pumping rate [96], locomotor activity [97,98], olfaction [99], gene expression, protein activity and regulation [100–103]. Bioinformatics analysis has identified *C. elegans* proteins that have high homology with the circadian clock proteins of other species. In particular, AHA-1, LIN-42b/c, KIN-20, and TIM-1 are considered putative homologs of mammalian CLOCK/BMAL1, PER, CK1ε/δ, and TIM, respectively [104,105].

4.2. Circadian rhythm disruption and its influence on physiology and metabolism

The pronounced effect of circadian rhythms on the regulation of key metabolic and physiological processes can be gauged by the fact that rhythmicity can be observed in the expression of nearly half (43 %) of the mammalian protein-coding genome [106]. Given the pivotal role of circadian rhythms in the maintenance of homeostatic periodicity, rhythm disruption adversely affects the physiology and overall health of an organism.

Disruptions in circadian rhythms occur either when the endogenous clock components and zeitgebers are out of phase, or when the master clock is not synchronized with the peripheral clocks [107]. Regular breakdown of circadian rhythms with a growing divergence between active and rest periods is a distinctive hallmark of circadian disruption [108].

Endocrine secretions such as cortisol, melatonin, prolactin, epinephrine, growth hormone, adrenocorticotropic hormone, thyroid stimulating hormone, ghrelin, leptin, and testosterone secretion regulate key physiological processes and are intricately controlled by the circadian clock [109]. The physiological maintenance of fluid flow, such as cardiac output, glomerular filtration rate, lymphatic flow, and vascular resistance, tends to follow strong circadian regulation. Diurnal variations in energy and nutrient demands are optimally matched by an equivalent circadian expression of ion channels and metabolic enzymes [110–112]. Core clock genes directly or indirectly regulate several intermediates of key energy homeostatic pathways, such as glycolysis, gluconeogenesis, glycogenesis, oxidative phosphorylation, lipid biosynthesis, and metabolism [113,114]. Perturbations in these pathways disrupt energy homeostasis, leading to diverse neurodegenerative and cardiometabolic disorders [115,116].

Further research with consistent tissue-specific manipulations is needed to establish the relative roles of individual clock genes and their interacting circuits in physiology and metabolism.

5. Bidirectional relationship between circadian disruptions and susceptibility to neurodegeneration

Disruptions in circadian rhythms, including alterations in behaviour (e.g., abnormal sleep/wake cycles), biochemical profiles (e.g., reduced antioxidant production), and physiological processes (e.g., decreased levels of endocrine secretions), are among the initial symptoms of various neurodegenerative conditions and are thus increasingly viewed as harbingers of neurodegeneration [117]. However, emerging evidence points to a bidirectional relationship between neurodegeneration and circadian dyshomeostasis [118,119]. In patients with neurodegenerative symptoms, the sleep/wake cycle and rhythmic expression of clock genes are disrupted, indicating the possibility of neurodegeneration-driven disruption of circadian rhythms [120].

The molecular clock components drive the rhythmic expression of genes and regulate post-transcriptional and post-translational processes, which may directly or indirectly influence the formation and transmission of misfolded protein aggregates [121–124]. The clearance of these aggregates occurs through sleep-based regulation of glymphatic flow [125]. Several studies have identified extensive daytime sleep, abnormalities in sleep behaviours, and daytime activity fragmentation, as consistent predictors of cognitive impairment and neurodegeneration [126,127].

Among the primary causal factors of neurodegeneration is oxidative stress. Disruptions in circadian rhythms contribute to neurodegenerative conditions by affecting the cellular response to oxidative stress [128]. Core clock genes such as *Bmal1* regulate antioxidant response elements [129]. Knockouts of the *Bmal1* gene in mice exhibit significantly increased oxidative damage compared to wild-type controls [130]. The rhythmic release of melatonin, a strong free radical scavenger, can also be considered part of the circadian control of the oxidative stress response [131].

Prolonged disruption of circadian rhythms leads to a wide variety of health issues owing to a generic imbalance in the homeostatic periodicity of physiological processes, resulting in changes in gene expression and inflammation. These effects may exacerbate the onset and progression of neurodegenerative pathologies [117,132].

Abnormalities in clock genes expression are observed in individuals suffering from various neurodegenerative conditions as well as in animal models of NDs. In AD, the temporal phase of the *Bmal1* expression varies from that in healthy controls [133]. Even the rhythms of *Bmal1, Cry1* and *Per1* transcripts are completely lost in the pineal gland [134]. In PD, *Bmal1* and *Per1* transcription and translation rhythms are blunted in several brain regions [135,136]. Similarly, in rodent models of HD, the regular rhythmic expression of *Per2* mRNA is disrupted [137].

The light/dark cycle is the primary zeitgeber for the circadian master clock present in the SCN. Recent evidence suggests a neurodegeneration-driven loss of intrinsically photoreceptive retinal ganglionic cells expressing melanopsin [138]. This leads to a reduced ability of light, as an external stimulus, to reset the circadian clock, ultimately leading to increased cycle-to-cycle variability and circadian dyshomeostasis.

The master clock in the SCN conveys temporal information to peripheral clocks present in cells and tissues through dedicated autonomic neural circuits and endocrine secretions. Specific cell populations within the SCN are vulnerable to AD-driven degeneration. Neurodegenerative states thereby alter the master-clock-driven rhythmic outputs in melatonin and cortisol secretion thus disrupting the synchrony of the molecular clocks present throughout the body [139].

6. Chronotherapeutics

Chronotherapeutic strategies are primarily based on three core approaches: (a) *Training the clock* to calibrate to a robust circadian rhythm, (b) *Drugging the clock* with molecules that target the circadian clock, and (c) *Clocking the drug* by developing a personalized regimen for drug administration [140].

Chronotherapy is practiced either by manipulating the sleep/wake cycles to improve sequels of pathologies or by observing the circadian pattern of an individual to develop appropriate and personalized therapeutic regimens. Organisms develop characteristic chronotypes and possess heterogeneous phases of endogenous clocks owing to diverse internal factors and external circumstances. Minor disruptions in the circadian cycle can affect the sleep/wake physiology, leading to various debilitating disorders. Chronotherapeutic strategies help restore the circadian cycle by incorporating proper sleep hygiene, adequate light exposure, physical activity, and chronobiotic medications, such as melatonin, in timed regimens [141].

6.1. Training the clock to avert human neurodegenerative conditions

Chronotherapeutic interventions may improve neurodegenerative disease states by restoring the circadian homeostatic periodicity. In the past, several efforts have focused on improving circadian output mechanisms and enhancing cellular oscillatory ability by targeting SCN oscillator populations [142,143]. In particular, several chronotherapeutic approaches simply function by facilitating central clock entrainment and the corresponding cellular clock synchronization [144].

Non-pharmacological approaches are regarded as the first-line of chronotherapeutics for managing circadian dysfunction and associated neurodegenerative conditions. These include bright light therapy, physical activity, and non-invasive transcranial stimulations using magnetic fields and low/mild electric current. To date, these approaches have been shown to be very effective in clinical settings [4]. Nevertheless, this review will focus on pharmacological approaches.

6.2. Drugging the clock – pharmacological chronotherapeutics to manage human neurodegenerative conditions

Chronobiotic substances adjust the timing of endogenous clocks to re-synchronize circadian rhythms. Clock-modulating molecules alter the circadian phase, amplitude, and period to enhance clock-regulated output mechanisms that may alleviate neurodegenerative conditions [3].

Melatonin, the first chronobiotic molecule, regulates the circadian rhythm by promoting sleep. The physiological levels of melatonin start rising at the end of the day to peak after midnight and decline before the end of the night. In general, it is naturally produced and released by the pineal gland under the control of the SCN to calibrate peripheral clocks. Melatonin and melatonin receptor agonists have been used to induce phase shifts in the circadian rhythm such as introducing a phase delay in the morning and a phase advance in the evening [145]. Clinical studies have demonstrated the benefits of melatonin in improving sleep quality in patients with AD and PD [146,147]. In HD and ALS patients, the efficiency of melatonin treatments has not been systematically investigated [4].

Several small-molecule clock modulators have been developed recently that can induce diverse physiological alterations. Some of these molecules work by either lengthening (e.g., CK1ε/δ inhibitors) or shortening (e.g., upstream kinases – ERK, phosphodiesterase 4, and adenylyl cyclase) the circadian period [148,149]. Another category of these molecules, such as lithium, acts by modulating the clock amplitude and period [150]. However, not all of these compounds have satisfactory bioavailability and pharmacodynamic properties. Therefore, *in vivo* animal studies and pre-clinical trials of most of these modulators have been a major challenge [148].

6.3. Clocking the drug – circadian rhythm as a potential target of neurodegenerative drugs

As mentioned, among the three broad categories of chronotherapeutic drugs, one refers to the optimization of the timing of the drugs, known as “clocking the drugs” [151]. This aspect is crucial in drug administration in general and especially in the case of treatments used in NDs that affect circadian rhythms, due to the bidirectional relationship between ND and circadian rhythm disruption.

In AD, donepezil (DNPZ), a cholinesterase inhibitor approved for the treatment of dementia [152], is transported in a circadian manner across the blood-cerebrospinal fluid barrier owing to the circadian expression of its membrane transporter ABCG2 [153]. In addition, DNPZ has been shown to affect sleep patterns and potentially influence circadian rhythms [154]. In the case of PD, studies have reported that the timing of levodopa (L-DOPA), a precursor of dopamine, significantly influences its efficacy. L-DOPA continuous administration leads to more stable plasma levels, reducing both the frequency and severity of motor complications such as dyskinesia [155]. Additionally, a study in rats showed that L-dopa reversed clock disruptions in PD models [156]. In the case of HD, tetrabenazine and deutetrabenazine, the most commonly used antidopaminergic drugs to treat HD-associated chorea, contribute to sleep and circadian rhythm disruptions [157]. In contrast, riluzole, a glutamate antagonist, benefits sleep in HD patients, with no relevant information available for ALS patients, for whom it is primarily used as a treatment [157,158].

Overall, the efficacy of drugs used for the treatment of ND may be influenced by circadian rhythms. However, the molecular pathways that

are affected by the timing of drug administration remain unknown and further preclinical studies in disease models ranging from *C. elegans* to mammals are needed to better understand this complex interplay. It is expected that "clocking the drugs" may improve their efficacy and reduce the occurrence and severity of side effects related to circadian rhythm disruptions, improving the quality of life of patients suffering from NDs.

7. Drug screening

Drug screening is a costly and time-consuming process that contributes to the transition of drugs from bench to bedside, utilising target- or phenotype-based drug screening approaches (TDS and PDS, respectively) [159], with TDS being the most widely used [160]. TDS begins with (a) target validation, the selection of an appropriate drug target based on previous studies, suggesting its involvement in the modulation of a disease of interest, or by *in vitro* and *in vivo* laboratory-based work, followed by (b) assay development to measure the activity of compounds against the target, and (c) low- or high throughput screening (HTS) that enables the selection of candidate molecule(s) for clinical trials [161]. However, the complexity of target validation is a major problem linked to TDS [162]. The second strategy, PDS, is based on screening for compounds that induce a certain phenotype without prior knowledge of the drug target. Drugs identified by PDS have been broadly used to reveal pathophysiological mechanisms [163]. However, the complexity of biological systems can increase variability and the mechanisms of action of certain compounds remain unclear. Thus, there is a need to translate these findings from *in vitro* studies to *in vivo* disease models before the initiation of clinical trials.

Various disease models, essential for the drug screening process, recapitulate a wide range of pathophysiological conditions [164]. Traditional two-dimensional (2D) cell culture has become keystone in drug screening, followed by the generation of more complex systems, including organoids, self-organising 3D structures [165], and organs-on-chip [166]. However, these platforms share a common limitation: the lack of complexity of multicellular organisms. This need for a whole-organism platform that mimics the characteristics of complex human diseases, such as ND, can be addressed using *C. elegans* [167]. Here, we summarise recent progress in the use of *C. elegans* as a drug screening platform, highlighting its advantages in HTS and drug discovery for NDs, as well as its potential application in chronotherapeutic drug screening.

7.1. C. elegans as a drug screening platform

C. elegans has been used as a model organism for genetic screening for approximately 50 years. However, its systematic use in chemical screening has only evolved notably since the early 2000 s, transitioning from small-scale candidate approaches to robust HTS [10]. Its key advantages, along with the large collection of multiple easily accessible strains from the *Caenorhabditis* Genetics Center (CGC) for several diseases, place *C. elegans* at the centre of drug discovery research [159].

Drug administration is an important variable in drug screening [168]. *C. elegans* is typically cultivated on plates or in a liquid medium [169], where large, synchronous animal populations can be easily maintained and studied for several days to weeks. In solid medium-based platforms, worms are cultured on nematode growth medium, and the drug can be mixed with the molten agar during preparation or added on top of the solidified agar later. However, this method consumes large quantities of compounds and is not amenable to HTS. In contrast, liquid-based formulations offer a significantly more manageable dosage setting [170].

Drug screening can be based on a combination of behavioural, morphological, and physiological tests to evaluate the effects of a compound [171]. Several behavioural assays are commonly employed to assess the effects of potential therapeutic drugs in *C. elegans* models of ND [66,172]. Locomotion defects are a common readout of PD and ALS in *C. elegans*, with alterations in crawling speed, body bends, and thrashing in liquid to be indications of damaged motor neurons or neuromuscular junctions [173,174]. Additionally, the olfactory-dependent chemotaxis assay, the ability of *C. elegans* chemosensory neurons (12 in the head and 2 in the tail) [175] to sense and respond to volatile organic compounds, is a powerful tool for evaluating memory-like behaviour in drug screening for NDs, such as AD [176]. The third most common behaviour studied in the context of ND is pharyngeal pumping, defects in which are intricately associated with AD. Some compounds have neuroprotective effects that enhance pharyngeal pumping [176,177]. Finally, egg-laying has been studied, particularly in the context of AD, with nematode disease models showing defective hatching [178].

Morphological assays are also performed to evaluate the effects of compounds on neuronal structure during neurodegeneration. These assays typically involve the expression of fluorescent proteins under neuron-specific promoters in order to observe changes in neurons of the worm, such as cell body rounding, dendritic alterations, and axonal degeneration [9]. Physiological assays, including calcium imaging, electrophysiology, and optogenetics, have also been used to evaluate the effect of drugs on neuronal activity [179]. However, monitoring neuronal activity is not ideal for HTS because of technical considerations and difficulties in optimizing conditions across multiple sessions [180]. The integration of behavioural, morphological, and physiological evaluations in *C. elegans* provides a comprehensive framework for screening compounds for therapeutic activity in worm models, which may have translational potential.

In HTS, implementing the appropriate controls is essential to ensure platforms' performance and detect potential variability between replicates or technical issues. This is similar to other drug screening methods [181,182]. Positive and negative controls should be included in drug screens to identify compounds that improve NDs. For example, rapamycin and dimethyl sulfoxide (DMSO) were used in a behaviour-based drug screening with a *C. elegans* model of motor neuron disease [181]. In the case of chronotherapeutic drug screening, melatonin [182] can be used as a positive control to validate assay sensitivity. Untreated or vehicle-treated worms can be used as a negative control to establish baseline activity and circadian rhythm profiles. Additionally, genetic controls, including circadian gene knockouts (e.g., *lin-42*, *tim-1* [104]) will contribute to the confirmation of clock-dependent drug actions.

Although *C. elegans* is widely used for drug screening, it has some drawbacks. First, despite having an innate immune system, it lacks an adaptive immune system and a typical inflammatory response [183]. Thus, studying the complex interplay of neurodegeneration-neuroinflammation is difficult. In addition, the relative inefficiency of drug uptake owing to the cuticle is also a major limitation in drug screening [184]. Finally, *C. elegans* uses *Escherichia coli* as its primary food source, which can metabolize and alter the availability of compounds when alive. This issue can be resolved by killing the bacteria, ideally through screening in a liquid medium with heat-inactivated bacteria [185].

7.2. C. elegans as a high throughput drug screening platform

After the initial experiments in drug screening using *C. elegans* in 1974 [186], several large-scale drug screens were performed to enhance the data throughput and analysis. HTS involves testing many compounds in an automated manner with the main aim to identify 'hits'. 'Hits' and 'leads', derived from the initial 'hits', are the compounds that affect the target in the desired manner at a sufficiently low concentration to maximize the specificity while minimizing the side effects [187]. Significant technological advancements in culture conditions and assay development over the past 20 years have enabled the utilization of *C. elegans* in HTS [10,188].

The first report on the use of *C. elegans* for large-scale drug screening was documented in 2006 [189]. In this case, 14,100 molecules were

tested in wild-type nematodes on agar, of which 308 compounds were identified as inducing a variety of phenotypes. While the distribution of worms was automated using a COPAS BIOSORT worm sorter (Union Biometrica), phenotypic analysis was scored manually, with results prone to bias and error over time [159]. In the same year, another study was published using liquid media to screen for antimicrobial compounds, making the use of *C. elegans* in HTS significantly more amenable [190]. Although these studies incorporated a level of automation, they also required careful manual phenotypic assessment of the worms.

The first system that introduced automated animal transfer, image acquisition, and data analysis was a significant step in the HTS history [191]. In this case, minor adjustments enabled the use of a primarily cell-based format, ArrayScan VTI, to capture entire wells with worms in one field. Since 2010, significant progress has been made in automating the analysis of complex phenotypes, such as locomotion, that can be used to study the effects of chronotherapeutic drugs in ND models. TheWormToolbox, which is available via the open-source CellProfiler project enables the scoring of worms using HTS [192]. A significant advancement in the automated image acquisition system, which allows for the quantitative analysis of four phenotypes assessed in *C. elegans* whole organism studies (mortality, movement, fecundity and size), came a few years later with the WormScan [193]. WormScan can be used in high-throughput analysis to identify potential chronotherapeutics with a holistic readout for survival and stress resistance in ND models (Fig. 2A).

Taking advantage of the complete automation of HTS, an ultra-high throughput screen was reported, which increased the throughput of *C. elegans*-based drug screening. This was achieved using 1536-well plates and automating animal transfer using the BioTek MicroFlo automated microplate dispenser [194]. However, for applications requiring specific animal size, number, and fluorescence intensity, the COPAS BIOSORT sorter offers an advantage, albeit at the cost of time [195]. Hence, COPAS could be used to identify drugs that may alter the expression of circadian rhythm-related genes, such as *lin-42* and *tim-1* [104], and potentially have neuroprotective effects (Fig. 2B).

Currently, microfluidic devices are used for *C. elegans*-based HTS, allowing automated worm handling and compound delivery with simultaneous or sequential observation [196]. However, the small size of worms and their constant movement pose a significant challenge to HTS accuracy. Hence, advances in immobilization, synchronization, and body orientation should be considered (Table 1) [197]. Imaging and screening at high resolution are crucial during HTS, especially in the context of ND, allowing observations from neuronal degeneration to the level of protein aggregation over time. Therefore, it is crucial to ensure worm immobilization without affecting the animal's physiological state to enable long-term monitoring of locomotion to study circadian rhythm (Fig. 2C). This has been achieved by various methods, including chemical, such as CO_2 [198], electrical, using electric field stimuli [199], mechanical, using a densely packed multi-channel device [200] and thermal, using droplets of thermosensitive hydrogel [201] methods. In addition, worm synchronization is particularly useful for studying age-related diseases such as ND. Typically, *C. elegans* synchronization is achieved by bleaching adult worms [202]. A recent method enabled

Table 1
Advances in microfluidic devices for high throughput screening in *C. elegans*.

Advancement-scope	Applications	References
Immobilization (chemical)	Appropriate for long-term (1–2 h) immobilization	[198]
Immobilization (electrical)	Movement-based microfluidic assays	[199]
Immobilization (mechanical)	Optical surgery, imaging and stimulating multiple worms	[200]
Immobilization (thermal)	Precise handling of early larval stage worms	[201]
Synchronization (via electrical sorting)	Movement-based assays with large number of synchronized animals	[203]
Synchronization (via rotatable capillaries)	Neuronal imaging (neuron-level assay)	[204]

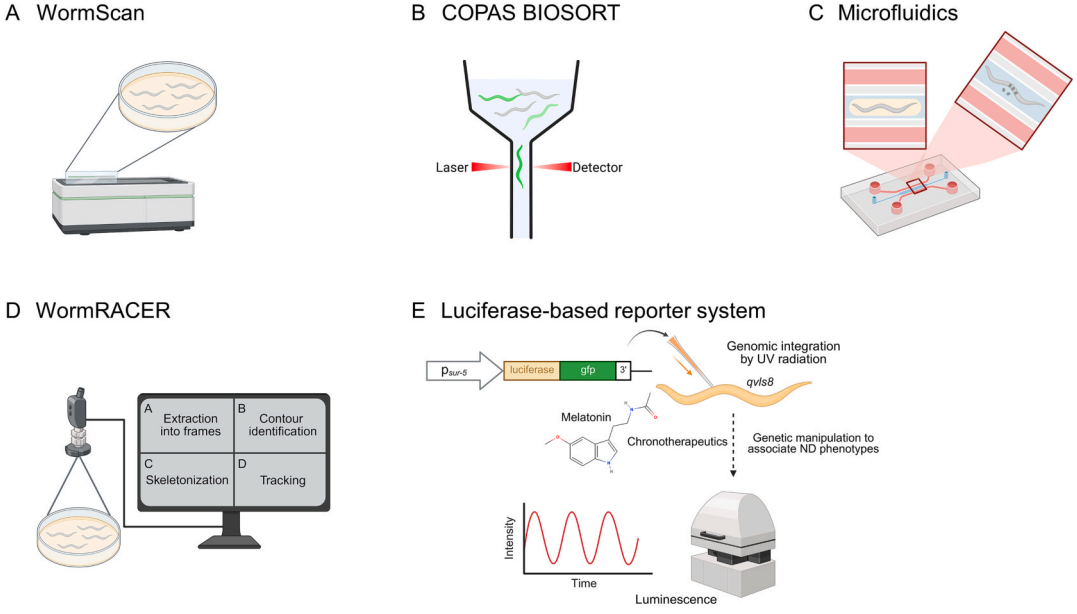

Fig. 2. Innovative strategies and techniques for screening and evaluating the efficacy of chronotherapeutic compounds in C. elegans. (A) WormScan could enable high throughput identification of potential chronotherapeutics by holistically assessing survival and stress resistance; (B) COPAS BIOSORT, a worm sorter based on phenotypic analysis, could be used for the identification of chronotherapeutics that modulate the expression of circadian rhythm-related genes; (C) Advances in microfluidics enable worm immobilization and egg-counting, which could be used for long-term monitoring of locomotion and egg-laying, respectively; (D) WormRACER for automated detection of nematode behaviour and time-dependent drug effects across individuals and time points (adapted from [209]); (E) Luciferase-based rhythmic reporter was developed and integrated into the nematode genome to study circadian pattern of gene expression. This strain can be used to screen for chronotherapeutic compounds at both the single-worm and population levels [100,102].

worm isolation and separation based on locomotion and electrical sorting, overcoming the time consumption of conventional methods [203]. In addition to immobilization and synchronization, worm orientation is a challenge, especially when imaging specific regions within the organism. Thus, a novel microfluidic device was developed to demonstrate the orientation of *C. elegans* larvae for multidirectional manipulation and imaging by integrating rotatable glass capillaries with pneumatic suction [204].

As described in HTS, multiwell plates or microfluidic devices are typically used, the former to provide a static fluid environment and the latter to offer flexibility in flow dynamics. However, by interfacing these two platforms in an automated manner, novel capabilities have been introduced, including the precise timing of drug administration [205]. In 2016, the first large-scale microfluidic device in a 96-well setup was developed, which tested ~ 1,000 FDA-approved clinical compounds in *C. elegans* models of HD, enabling worm immobilization with high-resolution imaging and automated data analysis [206]. Recently developed novel microfluidics devices can also contribute to ND assays and potentially enable experiments with precise timing of drug administration, which is a core requirement of chronotherapeutic studies. For example, the egg-counter offers a higher throughput compared to traditional solid-based assays and also provides a remarkably high degree of temporal resolution (Fig. 2C) [207]. In addition, microfluidic devices that immobilize only the head of *C. elegans* enable simultaneous recording of both neuronal activity and tail movement, providing information on brain activity in the context of movement [208]. Automated tracking systems, such as the Worm Robust Analysis by Computer-Enhanced Recording (WormRACER) [209], allow for the automated detection of worm behaviour and potentially time-dependent drug effects across many individuals and multiple time points (Fig. 2D). Further development of microfluidics will enable more complex *C. elegans*-based HTS revealing new 'hits' as potential therapies against neurodegeneration, while light- or temperature-sensitive systems will allow for a more accurate study of chronotherapeutics.

Although there have been several advances in HTS, several unmet challenges remain. The mobility and structure of worms complicate imaging and data analysis. This limitation can reduce the efficiency of HTS protocols, requiring specialised equipment, and increasing the cost of HTS assays [10]. Additionally, the most widely used microfluidics, made from polydimethylsiloxane (PDMS) devices, are autoclavable; however, it is challenging to operate devices under completely sterile conditions. Thus, the long-term study of worms increases the risk of contamination. Finally, a major limitation of microfluidics is the inability to recover the progeny from the worms used in the study. However, platforms, such as WorMotel [196], overcome these limitations, enhancing the *C. elegans*-based HTS efficiency, thereby advancing its throughput for preclinical drug discovery through *in vivo* assessment of thousands of compounds in a cost-effective and time-efficient manner.

7.3. *C. elegans as a promising model for chronotherapeutic drug screening*

Chronotherapeutic drugs can target circadian factors involved in maintaining neuronal physiology. This may help restore neuronal homeostasis, mitigate neurodegeneration, and potentially slow the progression of NDs. This signifies the relevance of testing these drugs in reliable and robust neurodegenerative disease models to investigate their efficacy in treating neurodegeneration [210,211].

Thirty-eight percent of genes associated with human diseases, including NDs, have orthologs in the *C. elegans* genome [13], making it one of the most suitable models to study these diseases and their associated phenotypes. For example, null mutants of *lrk-1* PD models [43] could be used to evaluate the rhythmicity of locomotion. Similarly, ALS worms with mutated *alfa-1* that exhibit age-related paralysis [57], can be used to investigate similar behaviours and screen compounds that may restore circadian function. Hence, these and other well-established models, as described in previous sections, could serve as robust platforms for the initial evaluation of potential chronotherapeutics in a neurodegenerative context. With the ongoing development of innovative technologies, there is growing potential to enable efficient and scalable testing of chronotherapeutics using C. *elegans* as an HTS platform (Fig. 3).

However, our understanding of the circadian rhythms in *C. elegans* is limited. Mutant animals with impaired rhythmicity can be used to screen drugs that could restore rhythmic behaviours, such as locomotion or gene expression. This process can identify potential chronotherapeutics, that could be further tested for improving ND conditions. Progress in characterising the circadian clock in these animals has been slow due to the lack of standard techniques for measuring daily rhythms [87]. This is the major limiting factor for testing chronotherapeutic drugs in *C. elegans.*

Nevertheless, circadian rhythms in worms can be measured because of the development of a locomotor activity-based recording system [212], followed by an imaging-based analysis system for long-term recording and analysis of *C. elegans* locomotion data [98]. The *C. elegans* transgenic strain VQ1310 *qvIs8*, which expresses a luciferase-based luminescent reporter under the control of the promoter of the suppressor of activated *let-60* Ras (*sur-5*) gene, has been developed to monitor circadian rhythms at both the population and single-worm levels. This strain can be entrained by temperature and light/dark cycles and can also be retrained upon phase shifts of the synchronising agents [100,102]. Subsequently, transgenic mutant strains can be developed to study nematode homologs of clock genes using the luminescent rhythmic reporter. These strains can also be used specifically for the discovery and screening of chronotherapeutics. In recent years, *lin-42* and *kin-20* mutant strains were generated using this technique to assess the circadian rhythmicity of these clock gene homologs in *C. elegans* [100] (Fig. 2E).

The development of a reliable bioluminescent reporter-based method has made it possible to obtain robust measurements of putative circadian gene expression under rhythmic and constant environmental conditions [102]. Thus, it is now possible to study daily rhythms in the production of melatonin [93]. This technique can be used to test the efficacy of melatonin and its receptor agonists in transgenic worms displaying neurodegenerative phenotypes. Similarly, other clock-modulating drug candidates can be tested for their effects on the maintenance of neuronal physiology and amelioration of neurodegenerative disease states. The *C. elegans* ND models expressing human amyloid beta, tau, or α-syn, and displaying ND phenotypes can be genetically manipulated to express the luminescent based rhythmic reporter. These transgenic strains can be further exploited to test the efficacy of chronotherapeutic drugs in ameliorating ND phenotypes. Simple genetics, high fecundity, higher reproducibility rate, and the possibility of following chronotherapeutic drug kinetics in a high throughput assay make *C. elegans* a versatile platform to screen chronotherapeutic drugs for NDs.

Despite the apparent benefits of using *C. elegans* for chronotherapeutic drug screening, there are several pitfalls to consider. *C. elegans* is a relatively simple organism that lacks many complex physiological systems [11]. Thus, initial findings need to be validated in mammalian models that more accurately recapitulate circadian rhythms compared to nematode models. In addition, drug metabolism in worms differs significantly from that in mammals. Therefore, pharmacokinetics and pharmacodynamics in *C. elegans* may not be effectively predicted, complicating the "clocking the drug" process that could improve a drug's efficacy. The short lifespan of nematodes, cannot provide information on the long-term effects of chronotherapeutic interventions, especially those with cumulative or delayed effects over time. Thus, while *C. elegans* is a valuable model for initial discoveries in circadian rhythm and chronopharmacology due to its numerous advantages, its limitations require careful consideration when translating the findings to humans.

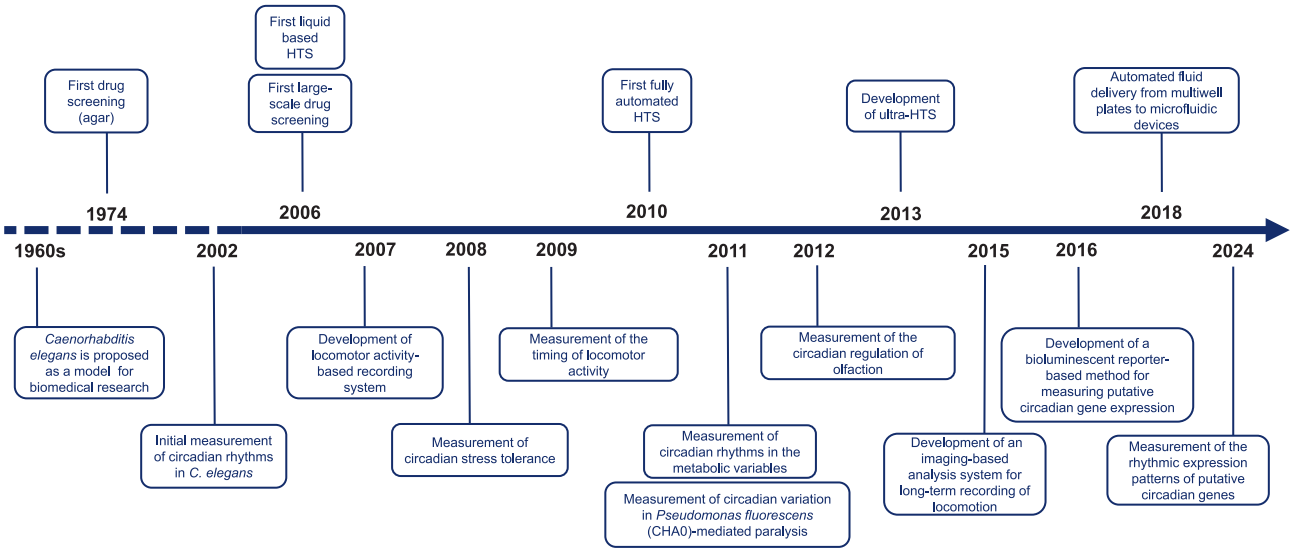

Fig. 3. Historical timeline of the advances in drug screening, and the measurement of circadian rhythms in *C. elegans*.

8. Concluding remarks

In the modern world, we frequently make deliberate choices, often out of necessity, to override our innate circadian cycles. This can result in biorhythms becoming dissonant with nature. When eating and sleeping patterns conflict with the natural alignment of the light/dark cycle, they cause circadian misalignment and disrupt physiological homeostasis. Regular disruption of the circadian cycle can lead to adverse health effects. This imbalance is associated with various health problems, ranging from metabolic disorders to neurodegenerative diseases.

NDs are a major global health concern. With the increasing average age of the population, the need to treat age-associated NDs has become pertinent. Our current understanding of the circadian control of neurodegeneration is minimal. Systematic investigations can significantly advance our understanding of perturbations in clock mechanisms that mediate neurodegeneration. This will lead to the identification of new clock modulating drugs that can ameliorate ND pathology.

There are numerous challenges in discovering new drugs or repurposing existing drugs for the treatment of NDs. Chronotherapeutic drugs are effective in managing several neurodegenerative conditions. However, the transition from bench to bedside requires strict preclinical and clinical trials to test the efficacy and safety of the compounds involved. From this perspective, transgenic *C. elegans* models offer a powerful, ethically simple platform for studying the interplay between circadian rhythms and neurodegeneration. A pipeline combining behavioural monitoring with morphological assays and life-history traits (lifespan, reproduction) under time-controlled dosing can serve as a high throughput chronotherapeutic screening platform. Thus, *C. elegans* can be used as a versatile preclinical drug testing platform for high throughput screening of lead compounds with potential chronotherapeutic effects to maintain proper neuronal physiology and ameliorate neurodegeneration.

CRediT authorship contribution statement

Mrutyunjaya Panda: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Maria Fakitsa:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Maria Markaki:** Supervision, Writing – review & editing, Validation. **Nektarios Tavernarakis:** Supervision, Writing – review & editing, Validation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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