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Sex bias and omission exists in Batten disease research: Systematic review of the use of animal disease models

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ABSTRACT

Batten disease, also known as the neuronal ceroid lipofuscinoses (NCL), is a group of inherited neurodegenerative disorders mainly affecting children. NCL are characterised by seizures, loss of vision, and progressive motor and cognitive decline, and are the most common form of childhood dementia. At least one type of Batten disease and three types of mouse disease models show sex differences in their severity and progression. Scientific research has a recognised prevalent omission of female animals when using model organisms for basic and preclinical research. Sex bias and omission in research using animal models of Batten disease may affect understanding and treatment development. We conducted a systematic review of research publications since the first identification of NCL genes in 1995, identifying those using animal models. We found that <10 % of these papers considered sex as a biological variable. There was consistent omission of female model organisms in studies. This varied over the period but is improving; one third of papers considered sex as a biological variable in the last decade, and there is a noticeable increase in the last 5 years. The wide-ranging reasons for this published sex bias are discussed, including misunderstanding regarding oestrogen, impact on sample size, and the underrepresentation of female scientists. Their implications for Batten disease and future research are considered. Recommendations going forward support requirements by funders for consideration of sex in all stages of experimental design and implementation, and a role for publishers, families and others with a particular interest in Batten disease.

1. Introduction

1.1. Batten disease

Batten disease is the collective common name for the neuronal ceroid lipofuscinoses disorders (NCL), the most prevalent group of neurodegenerative disorders in children. Named for the British paediatrician Frederick Batten, who described the disorder in two sisters [1], NCL are clinically characterised by the progressive decline of motor, cognitive and visual functions, and are life-limiting. They are rare autosomal recessive, lysosomal storage disorders that typically present in early childhood, and are currently incurable [2]. Up to 13 distinct genetic forms are grouped together as NCL due to their phenotypic similarities and lysosomal accumulation of autofluorescent storage material (ASM) in the brain and throughout the body [3,4].

Incidence and prevalence rates for NCL are not comprehensive and there is no suggestions of a difference in incidence between males and females [5]. However, sex differences in their manifestation is observed in patients with CLN3 disease and in mouse disease models for three genetic forms. For humans with CLN3 disease, and for mouse models of CLN3, CLN6 and CLN8 diseases, females experience a more severe course of the disease than males. Other NCL genetic types may also show sex differences in their manifestation but this is not reported. We describe the known sex differences briefly, why this may occur and why it is important to take into account, before using a systematic review to examine whether sex differences are considered in research design for Batten disease, especially that using model organisms.

Here, we use the following definitions of sex and gender [6]. Sex

Abbreviations: ADR, adverse drug reaction,; ASD, autism spectrum disorder; ASM, autofluorescent storage material; CNS, central nervous system; DEG, differentially expressed genes; ECG, electrocardiogram; EPMR, progressive epilepsy with mental retardation; GalCer, galactosylerceramide; mGluR5, metabotropic glutamate receptor 5; MS, multiple sclerosis; NCL, neuronal ceroid lipofuscinosis/es; NIH, National Institutes of Health; PAH, pulmonary arterial hypertension; PK, pharmacokinetics; PD, pharmacodynamics; PLX3397, pexidartinib; SABV, sex as a biological variable; SOD, superoxide dismutase.

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refers to biological and physiological aspects of a person or animal model, which is generally assigned at birth on the basis of anatomy as male or female. In contrast, a human individual self-identifies their own gender which can include girl, boy, woman, man, and other identities, which vary in different societies and can change over time.

1.2. Sex differences in batten disease

Juvenile CLN3 disease, often referred to as Juvenile NCL, is the most common form of NCL, affecting about half of all patients diagnosed with NCL [4]. Symptoms typically present between 4 and 8 years old with death in the third decade. Affected children first experience loss of vision, then deterioration of motor function and cognitive ability as well as seizures and psychiatric symptoms. 80–85 % of CLN3 cases are homozygous for a deletion of 966 b (termed the 1 kb deletion) that removes two exons of the gene (c.461-280_677+382del) [7–9]. Most of the remaining cases of juvenile CLN3 disease are compound heterozygotes for this 1 kb deletion and another mutation in the same gene [8]. There are a few examples of less severe disease caused by mutations in *CLN3*, such as childhood visual failure without immediate further symptoms and visual failure with onset in adulthood [4]. These rare subtypes have not been specifically studied for sex differences. *CLN3* encodes an intracellular integral membrane protein whose function is not fully understood but which is involved in many aspects of cell biology [3,4].

Differences in disease onset and progression have been described in several studies of juvenile CLN3 disease. These are summarised in Table 1. A comprehensive study comparing the symptoms of females and males with juvenile CLN3 disease living in the USA found a difference in age of onset, progression, chronology and age at death [10]. The later onset coupled with an earlier death suggests a more severe course of the disease in females. The disease course overall also varied. Behavioural symptoms manifested earlier in males, whereas females lost intelligible speech and independent gait one year earlier than their male peers; 10 years after disease onset it was clear that females lost functional capabilities much faster than males. A study of the hospital records on 35 Danish patients with juvenile CLN3 disease born between 1971 and 2003 found similar results [11]. Although there was no significant difference between sexes in the onset of initial symptoms, females were diagnosed later, which suggests that other symptoms that might have prompted diagnosis of NCL were appearing later; females also faced a faster decline and died significantly earlier than males. A study of 27 Finnish patients with juvenile CLN3 disease suggests that females are more prone to psychiatric disturbances than males [12]. 74 % of patients experienced some level of psychiatric disturbance, with females significantly more likely to be affected than males. Females had a higher incidence of anxiety and depression as well as attention and social disorders; these are linked to disease progression and are a recognised feature of the disease. An earlier study researching the efficacy of antidepressants in juvenile CLN3 disease had also found that females are significantly more likely to be treated using psychotropic drugs than males, outnumbering male patients by 6:1 [13]. These proportions are more than the sex differences between different types of psychiatric disturbances in the general population [14]. Differences in cardiac function occur in several animal models of NCL [15]. In the late stages of CLN3 disease cardiac problems are common [16], with adult patients often experiencing a slowing heart rate with age, compared to healthy peers. Sex differences exist, as half the females (four of eight) recorded T-wave inversions in an electrocardiogram (ECG) before 19 years of age whereas none of the nine males showed issues until after age 20 years.

Sex differences have also been identified in mouse models of CLN3 disease (Table 2). The behaviour and motor ability of two of the four *Cln3* mouse models were compared to see which most resembled classic human juvenile CLN3 disease [17]. $Cln3^{\Delta ex7/8}$ [18] was designed to mimic the 1 kb deletion, and $Cln3^{-/-}$ was designed as a knockout [19]). Care was taken in directly comparing these mice as they were derived on different genetic backgrounds ($Cln3^{\Delta ex7/8}$ is outbred in 129Sv/Ev, $Cln3^{-/-}$ is in C57BL/6 J) due to metabolic, neurological and behavioural differences between laboratory mouse strains [20]. Female and male mice for both strains on both genetic backgrounds were compared at three ages (1, 3 and 6 months); complex differences were found [17], with the background strain contributing to these. Differences between the sexes varied according to the test. Giving one interesting example, 3-month-old $Cln3^{\Delta ex7/8}$ females on both genetic backgrounds and 6-month-old $Cln3^{\Delta ex7/8}$ (129S6/SvEv) females could stay on the rotating rod longer than males and $Cln3^{-/-}$ mice, and surprisingly also longer their WT counterparts. Overall, it was concluded that for these two models male $Cln3^{-/-}$ mice showed the most severe disease.

Sex differences have also been identified in other NCL mouse models, notably for CLN6 and CLN8 diseases (Table 2). There are no reports examining differences between human males and females with any type of CLN6 or CLN8 disease. The function of these membrane proteins are not fully understood but CLN8 plays a role with ER protein CLN6 [21] in

Table 1
Summary of sex differences in human CLN3 disease patients.

Disease pathology	Sex differences		Reference
	Female	Male	
Age of first symptom	6.2 years	5.2 years	[10]
Age of diagnosis	7.9 years	7 years	[11]
Age of first epileptic seizure	9.9 years	11.2 years	[11]
Age received gastric feeding tube	19.8 years	24 years	[11]
Age dependant on wheelchair for daily use	17 years	20.2 years	[11]
Cardiology	Experience T-Wave inversion <19 years	Experience T-Wave inversion >20 years	[16]
Age of death	20.9 years	22.2 years	[10]
Order of symptom onset	Vision loss, cognitive impairment, seizures, behavioural symptoms, motor symptoms.	Vision loss, behavioural symptoms, cognitive impairment, seizures, motor symptoms.	[10]
Psychiatric disturbances	More anxiety and depressive symptoms; more attention problems; more likely to be treated with psychiatric medication.	Less anxiety and depressive symptoms; less attention problems; less likely to be treated with psychiatric medication.	[11–13]

Table 2
Summary of sex differences observed in mouse models of NCL disease.

NCL	Mouse model	Sex differences	Reference
CLN1	$Ppt1^{-/-}$	Males show higher responsiveness to Pexidartinib (PLX3397) treatment, resulting in a more positive treatment outcome for male mice.	[32]
CLN3	$Cln3^{-/-}$	Complex sex differences in behavioural and motor tests.	[17,83]
	$Cln3^{\Delta ex7/8}$	Complex sex differences in behavioural and motor tests. Sex different response to Galactosylerceramide (GalCer) treatment, different gene pathways affected.	
CLN6	$Cln6^{nclf}$	Complex differencesin brain pathology. Females show more severe behavioural deficits, a faster motor coordination decline and die at an earlier age.	[32]
CLN8	$Cln8^{mnd}$	Females show faster retinal degeneration.	[27]

the transport of newly synthesised soluble lysosomal enzymes from the endoplasmic reticulum to the Golgi [22,23]. There are two quite distinct severity types of disease defined by mutations in the gene *CLN8* [24]. There is the severe late infantile CLN8 disease form which probably lacks CLN8 function and the less severe form associated with missense mutation p.(Arg24Gly) [25] where there is probably some remaining CLN8 function. Late infantile CLN8 disease presents between the ages of 2 and 7 years and those affected experience frequent seizures and rapid cognitive decline, with loss of the ability to speak, move and eat unaided. Complete vision loss is common, and most survive until their late teens [26]. The less severe form, referred to as Northern Epilepsy or progressive epilepsy with mental retardation (EPMR), is characterised by seizures and a mild decline in cognitive function beginning after the age of 5 [25]. The seizures take place frequently during adolescence but become less common with age, and these patients survive into late adulthood. Sex differences have been reported in the Cln8^{mnd} mouse model with female mice showing a faster rate of retinal degeneration [27] (Table 2). The average retinal degeneration of a 4-month female mouse was comparable to that of an 8-month male mouse. Female mice suffered atrophy and complete loss of the inner layers of photoreceptors significantly earlier than males with rod photoreceptors disappearing earlier. There was increased rate of oxidation in female retinas and an earlier increase in levels of antioxidant enzyme superoxide dismutase (SOD). However, unusually the mutant mice in this study were on the B6KB2 background. There have been no reports of sex differences in other aspects in the mouse model for CLN8 disease.

There are two distinct forms of CLN6 disease; a paediatric form caused by loss of CLN6 function, and a less severe adult-onset disorder originally described as Kufs disease type A, where partial CLN6 function is retained. Symptoms of the classic late infantile CLN6 disease present between the ages of 1.5–6 years, and include seizures, language impairment, motor and cognitive deterioration, and vision loss, with death before the age of 20 years. Patients with Kufs disease can present symptoms well into adulthood, at a typical age of 30 years (range teenage to >50 years); and there is no loss of vision in adult CLN6 disease [28]. There are several animal models for CLN6 disease including mice and sheep [29,30]. The *Cln6*^{nclf} mouse carries a spontaneous 1-bp insertion mutation in the *Cln6* gene that is analogous to one of the human *CLN6* mutations causing late infantile CLN6 disease [31]. Only one study has explored sex differences in CLN6 disease progression, which followed *Cln6*^{nclf} mice and control mice on the same C57BL/6 J background (Table 2). The pathology of the brain and behaviour was studied; at 2 months males had a higher level of ASM in the brain somatosensory cortex and at 6 months females had much more in the ventral posteromedial and ventral posterolateral nuclei of the thalamus. Behaviourally, females consistently performed worse in a variety of tests for spatial learning and memory from 6 months, and died an average of one month earlier than their male counterparts. Thus, female *Cln6*^{nclf} mice show a more severe course of the disease, with both earlier onset of symptoms and faster disease progression.

It is notable that observed sex differences have so far been described in NCL caused by mutations in membrane proteins. Sex differences in pathology or in animal NCL disease models has not been studied routinely. However, sex differences to a potential neuroinflammatory treatment has been reported in the response of the CLN1 disease mouse model, *Ppt1*$^{-/-}$, but not of control mice on the same C57BL/6 J background [32] (Table 2); PPT1 is a lysosomal enzyme.

2. Explanations for sex differences in batten disease

Many major diseases, such as cardiac, cancer, Alzheimer's [33] and infectious diseases such as COVID-19 [34] show sex differences. The reasons underlying the sex differences in NCL are unknown. There are multiple theories for why sex differences are observed in Batten disease.

2.1. Sex hormones

Possibly the theory with the strongest backing of the cause of sex differences in Batten disease is hormonal. Females with CLN3 have increased acne, hirsutism and hyperandrogenemia compared with healthy females of the same age [35]. This suggests a hormonal involvement in disease pathology. Furthermore, CLN3 females have a lower average age of menarche, experience irregular menstrual cycles and have a higher incidence of polycystic ovary syndrome. The effects on menstruation suggest the involvement of oestrogen in the disease. This is relevant to neurodegenerative disorders, since receptors for oestrogens and oestrogen-like molecules are found ubiquitously and usually considered to have a protective effect [36]. For example, females with Parkinson's disease experience a less severe course of the disease. However, when oestrogen, specifically endogenous oestradiol, decreases in females their symptoms typically become more severe [37]. In the NCL oestrogen may be having the opposite effect on patients. Females' physical disease progression begins to overtake their male counterparts as they hit puberty when oestrogen is increasing. Moreover, the more severe psychotic symptoms usually set in after age 13 and are often associated with earlier age of menarche [13]. There are no reports on basal levels or differences in circulating oestrogens in patients with NCL (or animal models). A study to look at the levels of circulating oestrogens, or whether there is reduced synthesis of oestrogens or receptor downregulation, or abnormal or aberrant hormonal signalling in CLN3 disease, would address this theory and may open up new routes to therapeutic development.

A hormonal involvement in the faster female disease progression on *Cln8*^{mnd} mice (which is on the rare genetic background B6KB2) has been theorized [27]. The possibility of a hormonal involvement in the increased levels of SOD enzyme in female retinas was discounted after testing both healthy and mutant mice across the oestrus cycle. However, sex hormones influence the structural and functional organisation of the retina and previous studies have found sex hormone levels can affect pathogenesis in a range of ocular disorders [38].

Hormone differences in males and females could be affecting the rate of neurodegeneration through regulation of the brain's immune response, specifically the neuron-glia interactions [31]; glial cells perform a variety of roles including providing the immune response of the brain, and regulating neuronal communication and function. Despite their protective role, ongoing glial activation in the brain can cause the damage and death of neighbouring neurons. Patients with NCL experience chronic neuroinflammation that is sustained by persistent glial activation; this leads to the ongoing pathology of the CNS and causes neuron damage and death. Sex hormones are known to affect the coordination of neuron-glial cell interactions therefore the differing hormones in males and females could ultimately lead to differing rates of neuronal cell death [39]. Notably testosterone has been proven to decrease reactive neuroinflammation after neuronal cell damage [40].

Further, there is sex-biased modulation of autophagy though sex hormones and their receptors [33]. Autophagy, which is central to maintain cell homeostasis, has been shown to be affected in neurodegenerative diseases [41], including in studies of NCL and NCL model systems [42–51].

2.2. Immune system sex differences

There is neuroinflammation in NCL [52] and individuals with juvenile CLN3 disease raise autoantibodies against identified brain antigens. These autoantibodies are detected in the CNS of humans and model organisms [53,54] and theorized to contribute to the neuronal cell death as supressing this autoimmune response in a mouse model for Cln3 disease can slow progression [10].

There are known sex differences in many autoimmune disorders, and females have a much higher incidence [55], with women nine times more likely than men to be affected by systematic immune disorders.

Even in healthy immune systems there are sex differences; females have increased immunoreactivity, with more circulating antibodies and a higher number of T cells.

Sex different NCL immune responses could be further influenced by hormones, and the underlying cause for sex differences in the immune system may be hormonal [55]. For example, in systemic lupus erythematosus females with higher levels of oestrogen experience significantly faster disease progression [56]. Increased levels of oestrogen in females post puberty could be accelerating the autoimmune progression and therefore neurodegeneration injuvenile CLN3 disease [13]. There is some evidence of protective testosterone in autoimmune disorders, and there has been some success in artificially treating female multiple sclerosis (MS) patients with testosterone [57]. So, the increased levels of testosterone coupled with decreased levels of oestrogen could provide autoimmune protection in older males with juvenile CLN3 disease. In most other types of NCL (e.g. CLN1, CLN2, CLN5, CLN6, CLN7, CLN8 diseases) those reaching puberty are already severely affected.

2.3. Alternative physiological theories

It has been suggested that sex disparity could be the result of genetic differences between males and females [10], with the Y chromosome providing a degree of protection for males. This is known in several diseases including pulmonary arterial hypertension (PAH) [58], a chronic pulmonary vascular disease with a hallmark of dysregulation of cellular proliferation and apoptosis. PAH is significantly more common in women, and several genes only expressed on the Y chromosome may prevent dysregulation, thus providing protection for males who would otherwise develop the disease.

Males with a higher baseline muscle mass may have slower disease progression is simply as they have more muscle to lose [10]. Even prepuberty, males have a higher percentage of lean body mass [59]. This may explain why females become dependent on a wheelchair earlier and lose functional capabilities faster [10,11]. This theory alone seems unlikely as it does not explain the other symptoms with sex differences in their severity and progression, such as vision loss or psychiatric problems, that do not rely on muscular strength. Furthermore, this does not appear relevant to other muscle wastage disorders where males and female undergo similar pathological changes thought to be due to sex differences in the microenvironment and intrinsic signalling and requiring different treatment [60].

In the Cln3 mouse models [17] and perhaps other NCL mouse models, background strain genetics could be introducing or modulating sex differences in disease progression [20].

2.4. Societal theories

There could be a societal factor influencing NCL diagnosis and reported progression in patients. The earlier diagnosis of males with NCL could be due to the differing societal expectations on males and females [10]. Parents have higher physical expectations for young boys than they do for young girls. A study observing parental reaction to their children completing a physical task with a small amount of risk [61] found both mothers and fathers are more likely to encourage independence from sons. Despite both sexes showing the same level of competence parents were significantly more likely to assist their daughters. This phenomenon could be affecting the age of NCL diagnosis as parents may be more likely to notice typical NCL symptoms in males at a younger age as they encourage a higher level of independence. As a result, girls go undiagnosed longer. This theory is at least in part supported in the study of 35 Danish patients where there was a difference in age of official diagnosis yet no significant difference in the age of first symptom onset [11], with females diagnosed later than males. Differences in recognising symptoms in females due to societal expectations is not uncommon. Autism spectrum disorder (ASD) has historically always been considered a mostly male disorder, who are diagnosed at 4 times the rate of females [62]. However, it has also been suggested autistic females simply go undiagnosed [63] due to differing social expectations of young girls and boys. Diagnosis of ASD is more complex than for NCL as there is no genetic test and it relies on a cluster of observable symptoms.

Sex differences in NCL disease are not the result of societal influences as numerous studies show quantifiable physical differences between the sexes [16,31] and there is evidence for sex differences in brain structure in AD [64]. However societal gender expectations could be contributing to the later diagnosis of females with NCL. The study of mouse NCL models could provide an invaluable opportunity to isolate and stratify genetic, physiological, and societal components to diagnosis, and especially so if sex differences do not occur in all NCL models.

3. Sex differences in scientific research involving model organisms

There are sex differences in the pathology of NCL disease and in NCL model organisms, and the reasons for this are not understood. The study of NCL and theraputic development using animal models should therefore consider both sexes. However, in basic and preclinical research, a male mouse bias exists. Female model organisms are consistently excluded from research [65], and meta-analyses have found the routine omission of females throughout multiple disciplines. A significant sex bias was found in a landmark comprehensive review of papers published in 2009 from the major biological journals [66]. In ten biological fields, immunology, neuroscience, physiology, pharmacology, reproduction, endocrinology, behavioural physiology, behaviour, zoology and general biology, there was a significant male sex bias in all but two fields, reproduction and immunology. Moreover, the subject sex was frequently omitted. In immunology 60 % of papers failed to specify sex of their model organism. In terms of overt male bias, neuroscience was found to have the most uneven ratio where male-only papers outnumbered female-only by 5 to 1.

A more recent meta-analysis carried out regarding neuroscience research is consistent with these findings [67]. Reviewing all neuroscience research released in 2017 in the 6 major journals; Journal of Neuroscience, Neuron, Journal of Neurophysiology, Nature Neuroscience, Science and Nature, 26 % of papers were found to use only male model organisms compared with 5 % that included only female. Furthermore, 16 % failed to mention the sex of their model organism and only 8 % considered sex as a biological variable.

4. Investigation of sex bias using a systematic review of published scientific research involving model organisms for NCL

Here, we undertook a systematic review of papers published since the first identification of NCL genes to determine whether a male model organism bias has applied, and continues to apply, to the study of NCL. This approach follows the PRISMA principles [68,69] and the information flow is summarised in (Fig. 1).

4.1. Literature review search methods

4.1.1. Inclusion criteria and coding or articles

A representative sample of NCL primary research papers using model organisms published since the first identification of NCL genes (25 years from 1995) was obtained by searching PubMed and Web of Science databases in March 2022 looking at records from 1995 to 2020. The following search terms were used: “Neuronal Ceroid Lipofuscinoses” “Batten” “Ceroid” and “Neuronal Ceroid Lipofuscinosis”. Only records in english were considered. Records were individually assessed to determine whether or not they should be included (Fig. 1). No missed records were identified through reading the original set.

Papers were excluded if reviews, editorials, comments or similar non-primary research papers; for not being relevant to NCL research

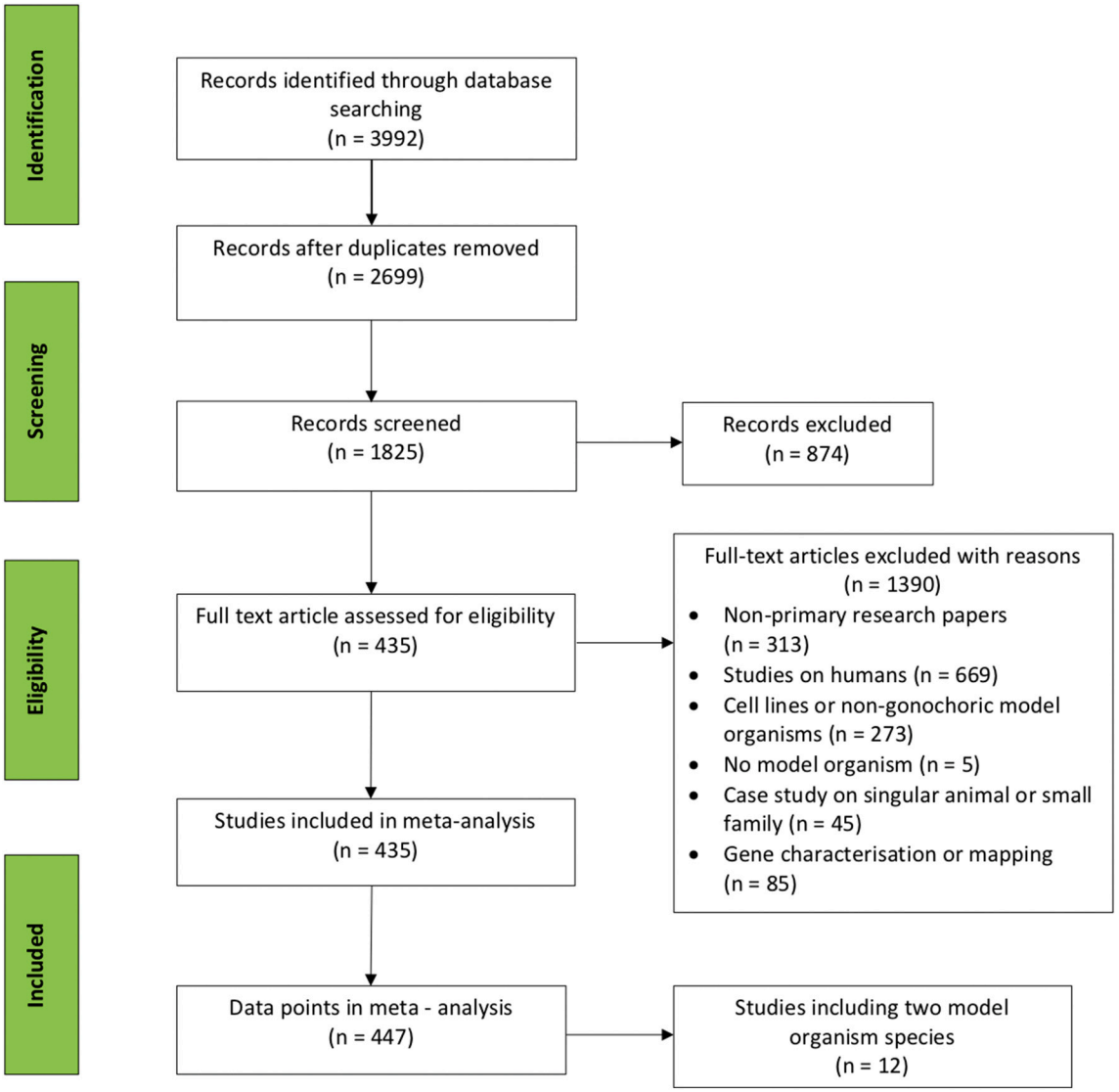

Fig. 1. PRISMA flow diagram of the study. This summarises information retrieved through the searching and selection process and data included in the study. PRISMA = Preferred Reporting Items for Systematic Reviews and Meta-Analyses.

including papers that were published by researchers with the surname Batten; studies carried out on humans (these have been summarised above); case reports describing the disease in either a single animal or a small family, where the researchers had no control as to the sex of the model organism/s. Research carried out on cell lines, foetuses or cell models were excluded as sexing, although important [70,71], may be impossible to do retrospectively. Papers were also excluded if the model organism was not gonochoric and gene mapping papers were not included. This resulted in 435 papers which were manually analysed to determine species and sex of the model organisms (Figs. 2, 3), as well as whether sex was included as a biological variable (SABV) (Fig. 3). SABV was considered to be included if the paper adhered to the 2016 National Institute of Health (NIH) SABV policy. This policy requires research to factor sex into the design, analysis and reporting of an experiment [72]. Thus, a paper was deemed to have included SABV if data for males and females was reported separately and compared. Simply "sex-matching" model organisms was not sufficient. Of the 435 papers included, 12 used multiple model organisms; the different species were considered as separate data points, making a total of 447. These are listed in the Supplementary Table, and can be independently sorted by each category. The dataset was analysed as a whole across the decade and then split into 5-year periods (1995[1996]-2000, 2001–2005, 2006–2010, 2011–2015 and 2016–2020) to analyse whether there has been any change across 25 years. 2016–2020 was further divided by year to analyse the potential impact of the introduction of the 2016 NIH SABV policy on animal model research. Results are displayed graphically (Figs. 1-7).

4.2. Results of the systematic literature review

Eight different research models were represented in the dataset (Fig. 2), with most being vertebrates. Mice were the most commonly used (75.4 %), followed by dogs (8.1 %), sheep (6.7 %), the fruitfly *Drosophila* (3.8 %), rats (3.1 %), non-human primates (1.3 %), zebrafish (1.3 %) and cats (0.2 %). Across the time period 1995–2020 there were 447 datapoints (Fig. 3). 54.8 % of papers did not state the sex of the model organism, 27.3 % used males and females, 15.4 % used only males and 2.5 % used only females. Of the 122 papers that included both males and females 36.1 % (44) included SABV, so overall 9.8 % of all papers in the 25-year period included SABV.

Breaking down the time period into 5-year intervals (Fig. 4), in most intervals the majority of papers did not state the sex (maximum was 74.7 % in 2006–2010 and minimum was 40.9 % in 2016–2020). Interestingly, of those papers using both males and females, consideration of

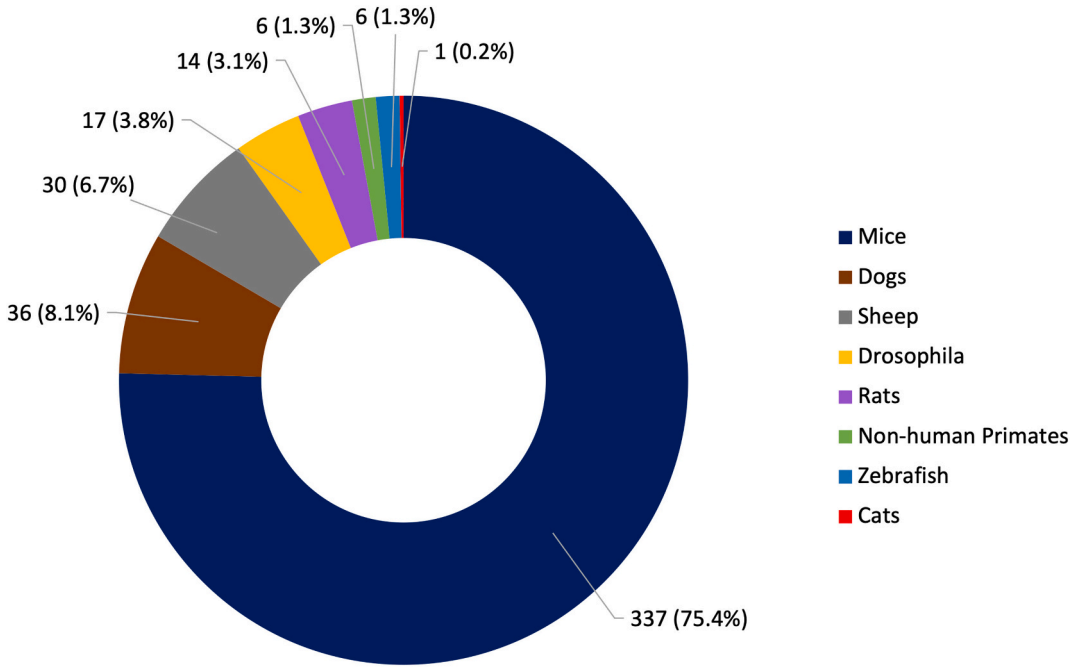

Fig. 2. The species of model organisms used in NCL research across the 1995–2020 timeframe. Number of data points and per centages are shown. Mice were the most common followed by dogs, sheep, *Drosophila*, rats, non-human primates, zebrafish and cats.

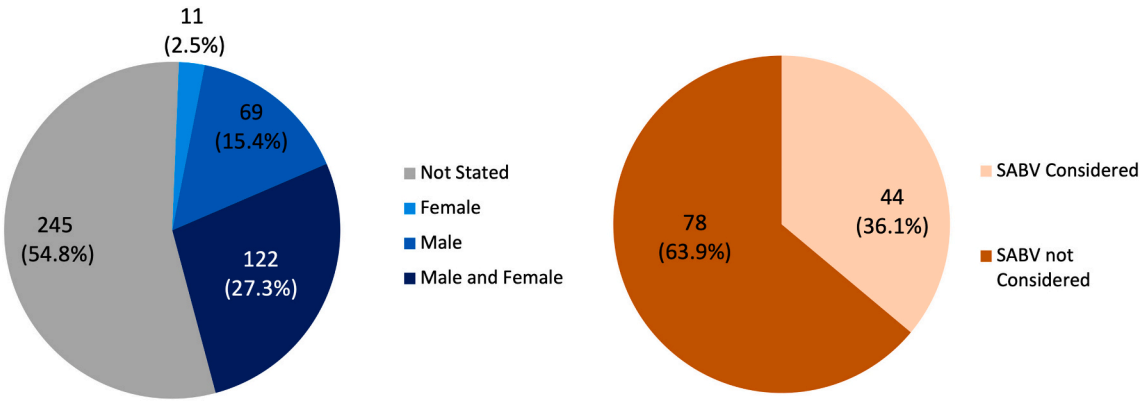

Fig. 3. Papers published between 1995 and 2020 in NCL research that used model organisms with sexes. (A) The % of published papers in NCL research stating the sex of animals used. (B) The % of published papers in NCL research including both male and female animals that considered sex as a biological variable (SABV).

SABV was highest in 2001–2005 at 63.6 %, dropped to 36.4 % in 2006–2010 and again to 29.7 % in 2011–2015 before rising to 37.0 % in 2016–2020. (Fig. 5). Papers defining the sex of animals used seems to be increasing from a low in 2006–2011. There was a decrease in the % of papers using only males (from 18.3 % in 2001–2005 to 12.6 % in 2011–2015), but this increased to 18.9 % in 2016–2020, suggesting that SABV is not yet established research culture.

Further analysis of the most recent 5-year time period of 2016–2020 does suggest an increase in consideration of sex. There has been a gradual increase every year in papers including SABV in this period from 2.7 % (1/36) in 2016 to 22.7 % (5/17) in 2020 (Fig. 6). This is possibly an impact of the 2016 NIH SABV guidelines.

4.3. Male mouse bias exists in NCL research

This analysis shows that the male animal bias does exist in NCL research, even in the most recent studies. Given that 75.4 % of animals used were mice, we looked in more detail at the reporting of the sex of mice, and found a male mouse bias. Across the 25-year period (Fig. 7) male-only mouse studies outnumber female-only studies by 6.4:1 (58:9). More than half the papers (54.6 % (184/337)) do not state the sex of the model organism used; it cannot be concluded that the mice used were predominantly male, although this would be in line with the observed bias. This omission does not show any signs of receding since the ratio of male-only studies to female-only studies was at 22:1 in 2016–2020 and papers not reporting the sex of the mice used remained high at 38.0 % in 2016–2020. However, in the two last 5-year periods the percentage of research reporting use of both sexes had increased to 29.1 % and 40.7 % respectively, up from a low of 8.7 % in 2006–2010.

5. Implications of the male animal bias in NCL research

This study highlights the lack of sufficient research on female model NCL organisms, exemplified in studies using mice which make up the vast majority of animals used in NCL research. There is a consistent failure to consider sex a biological variable despite the known sex differences in disease pathology which were first reported for NCL at least as early as 2001 [13]. This neglect could prove problematic for NCL

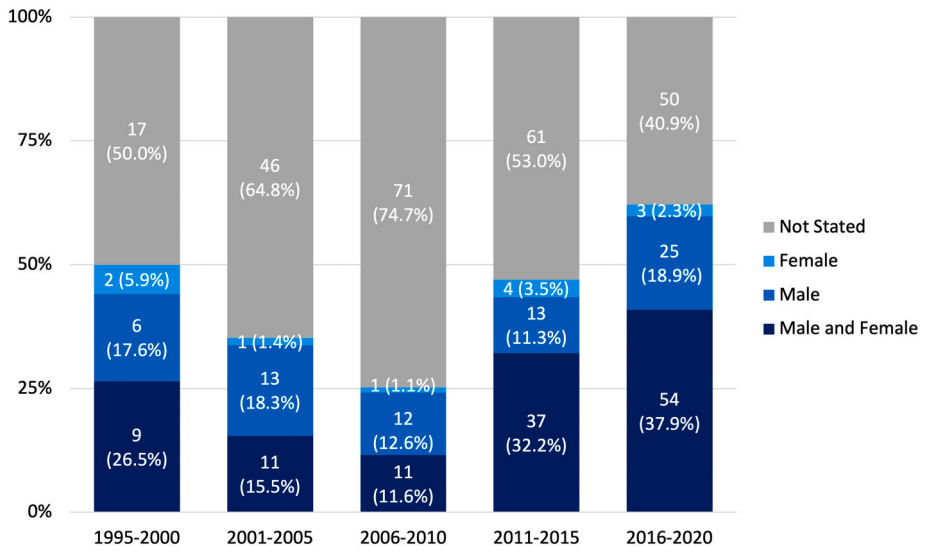

Fig. 4. Papers published between 1995 and 2020 in NCL research that used model organisms showing the distribution of sex bias and including failure to report the sex used. The number and % of published papers stating the sex of animals used shown by 5-year time intervals. There is a significant male bias as well as consistent failure to report the sex of organisms used.

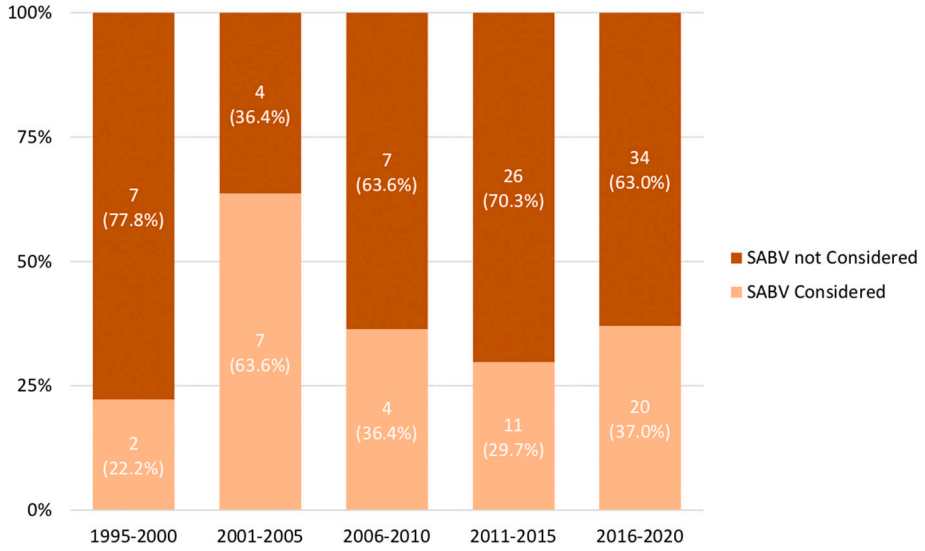

Fig. 5. Papers published between 1995 and 2020 in NCL research that considered sex as a biological variable. The number and % of published papers including both male and female animals that considered sex as a biological variable (SABV) of those papers, shown by 5-year time intervals.

research and the increased focus on treatment development. The implications of this omission for the NCL are considered in the following sections.

5.1. Sex differences in drug pharmokinetics and pharmacodynamics

Failing to thoroughly test potential therapeutics on both females and males is dangerous as there are multiple sex differences in drug pharmacokinetics (PK) and pharmacodynamics (PD). These have been documented in every stage of drug disposition [73]. They are the result of a combination of physical differences between the sexes. PK is affected by drug absorption and bioavailability, distribution, metabolism, and elimination and the cause of differences in PK include, but are not limited to differences in gastrointestinal motility, intestinal enzymes, body weight and percentage body fat, organ size, plasma volume, total volume body water and average organ blood flow, renal clearance including glomerular filtration rate. PD is concerned with the drug mechanism of action, which includes the physiologic and biochemical effects on the body, and the relationship between drug concentration and the rate and extent of the pharmacological response. Even at the same blood concentration, a drug may invoke variations in response, including differences in effectiveness or safety. PK/PD may be further influenced by the use of oral contraceptives, pregnancy and the menopause. Further, drug clearance relies on metabolic enzyme systems which have sex specific expression [73].

In a recent study of 86 drugs with known sex differences, 76 of these had a higher pharmacokinetic value as a result of slower absorption, metabolism and excretion in females compared to males [73]. One example is Zolpidem (Ambien), a commonly prescribed medication to treat short-term sleeping problems which in females has a blood concentration 50–70 % higher and a 35 % lower clearance rate, thereby remaining in the blood circulation for longer [74]. The recommended dosage was reduced by 50 % for females only in 2013, 25 years after first prescribing [75]. Drugs, such as betablockers and aspirin, produce a

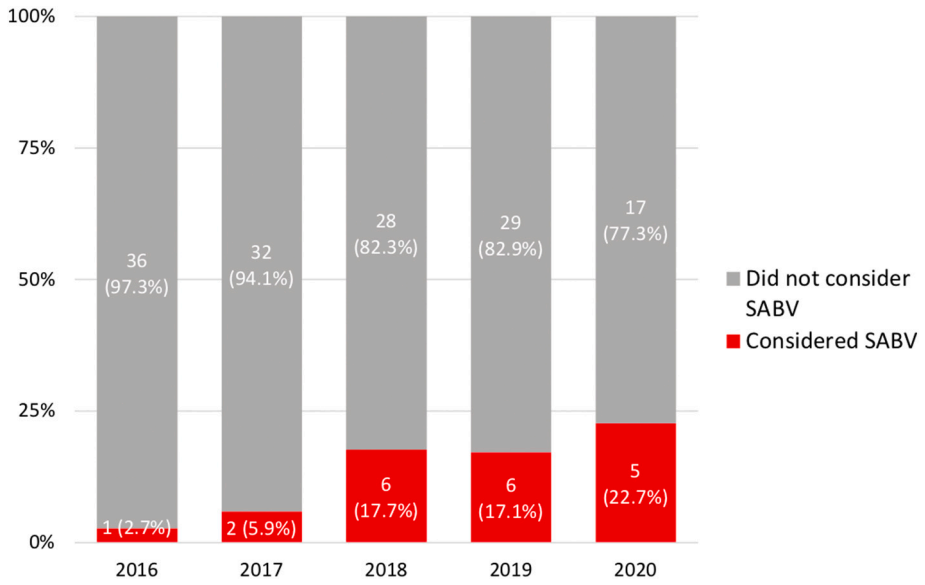

Fig. 6. Papers published between 2016 and 2020 using NCL animal models that considered SABV. The number and % of papers, shown by 1-year time intervals. There is a gradual increase by year in the per centage of papers that considered SABV in this recent 5-year period.

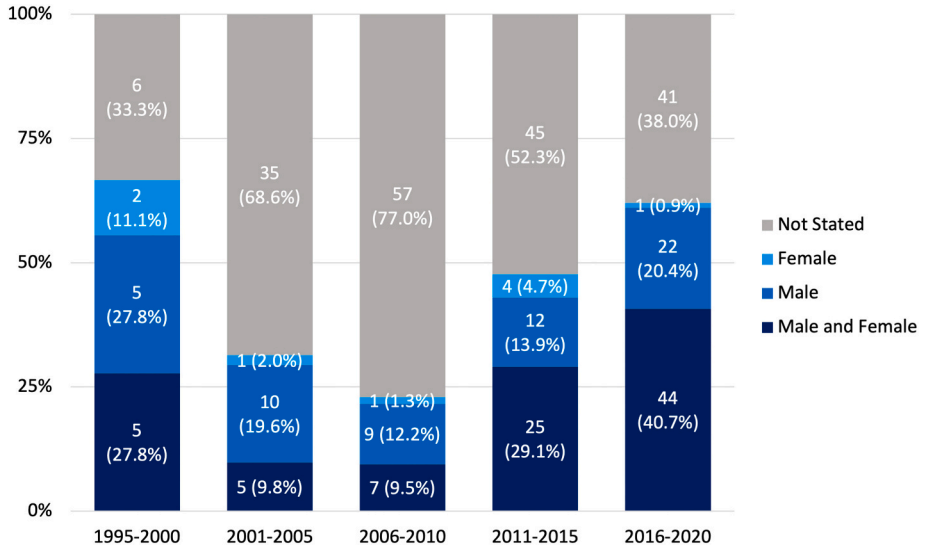

Fig. 7. Papers published between 1995 and 2020 that used mouse NCL models showing the distribution of sex bias and including failure to report the sex used. The number and % of papers stating the sex of mice used, shown by 5-year time intervals. There is a significant male bias as well as consistent failure to report the sex of mice used.

greater PD response in females. The sex differences in PK/PD and the lack of sufficient research including females can ultimately result in dangerous side effects. Females are nearly twice as likely to experience an adverse drug reaction (ADR) than males [73,76]. ADR range from nausea and headaches to cardiac anomalies and cognitive defects. Women are significantly more likely to be hospitalised as the result of an ADR.

It is important that sex differences in drug PK/PD is recognised and considered for the NCL. For most types of NCL there is currently no specific treatment available, and potential specific medication are mostly still in their early stages of development [2]. Nevertheless, patients are currently treated with drugs to alleviate symptoms, such as anti-epileptic drugs and anti-depressants [77]. Sex differences must be considered for any already prescribed drugs, and introducing the good practice of incorporation of sex differences in research from now on could prevent a higher incidence of future ADR in NCL females.

It is not just the potency and side effects of drugs that differ between the sexes. Some drugs do not work in both males and females, highlighting the need for sex-specific stratification in clinical trials. An overproduction of metabotropic glutamate receptor 5 (mGluR5) which reduces autophagy and the clearance of cellular debris, results in the accumulation of neurotoxic aggregates in the brain of humans and mice with Alzheimer's disease (AD). Pharmacological inhibition of mGluR5 activity was shown to mitigate β-amyloid pathology and reverse loss of cognitive function; however, these initial studies used only male mice. When female mice were subjected to the same treatment there was no improvement in pathology or cognitive function because the impact of pharmacological inhibition of mGluR5 activity on autophagic signalling varies between the sexes [78]. This is pertinent to the NCL, given that there are sex differences in autophagy [33,41], and that autophagy is affected in some types of NCL model systems [42–51].

5.2. Identification and understanding of sex differences can lead to insight

The discovery of a sex difference in diseases or drug efficacy could provide novel insight into disease mechanism. The study of MS found sex differences in both its incidence and severity [79]; females have a higher incidence, and males have a more severe pathology. Studying both male and female model organisms, and finding that pregnant female rats and guinea pigs exhibited protection from the disease, led to the discovery that in humans there is a 70 % reduction in MS relapses during the third trimester of pregnancy. This led to the hypothesis that oestriol, which increases in pregnancy, is protective, and current clinical trials are testing its efficacy as a possible treatment [80]. This potential treatment would never have been discovered if researchers had only studied male animal models. Interestingly, the drug tamoxifen was recently found to ameliorate cell phenotypes of cells deficient in CLN3 and CLN7 function, although the mechanism of action here seems to be independent of oestrogen receptor modulation and via the activation of TFEB, a key regulator of lysosomal function and autophagy [81]. Understanding the full basis for sex differences in the different types of NCL might open new avenues for therapeutic development.

5.3. The study of sex differences is important for NCL

Sex different responses to NCL treatment are not solely hypothetical. Two potential treatments were already found to have sex differences in NCL mouse models: GalCer treatment for CLN3 disease and PLX3397 treatment for CLN1 disease (Table 2).

Consistent treatment with the lipid galactosylerceramide (GalCer) showed improvement in the neurobehaviour of *Cln3*$^{\Delta ex7/8}$ mice [82]. GalCer exerts a positive impact on cell growth and increases longevity. Although both male and female *Cln3*$^{\Delta ex7/8}$ mice benefitted from GalCer treatment, when investigating the genes responsible for this efficacy sex differences were found [83]. There are 30 differentially expressed genes (DEGs) in female *Cln3*$^{\Delta ex7/8}$ mice treated with GalCer compared to control groups, and 66 in male *Cln3*$^{\Delta ex7/8}$ mice, with only one common DEG between males and females. This suggests that distinct pathways are being altered by GalCer treatment in male and female mice, which has implications in humans in terms of sex differences in ADR.

A 2020 study showed that long term treatment with Pexidartinib (PLX3397), a small selective inhibiter molecule, can decrease neuroinflammation in the CLN1 mouse model, *Ppt1*$^{-/-}$ [32], with a depletion in the number of pro-inflammatory glial cells, microglia, and improvement in motor coordination and visual acuity. There is however a sex disparity in its efficacy. Microglia in males show a higher responsiveness to the treatment and there is a more positive treatment outcome for male *Ppt1*$^{-/-}$ mice than females. This has implications in humans in terms of maximising efficacy for both sexes.

As discussed earlier, NCL have a neuroinflammation element [52] and CLN3 disease an autoimmune element [53,54]. The use of immunosuppressants has been explored with genetic and pharmaceutical approaches used to reduce the immune response in *Cln3*$^{-/-}$ mice, decreasing neuroinflammation and improving motor control [84]. However, this study was only carried out on male mice. Since male and female immune systems are significantly different, the failure to properly explore this in the early stages of therapeutic development could lead to key information being missed in terms of mechanistic understanding, or ineffectual or dangerous treatment for females being taken forward.

6. Reason for ineffective study of sex differences in model organisms

Researchers have known for decades that normal physiology and many diseases have significant sex differences and there has been a call for the inclusion of female model organisms in research for nearly thirty years [85], since before the identification of the first NCL genes. This has not happened, and several reasons can be attributed to the bias in use of male animal models.

6.1. Belief that females are more variable

It is a common assumption that male animals provide more reliable and less variable data overall, and that female animals produce more variable and therefore less reliable data. This is often linked with the female oestrus cycle with fluctuating levels of circulating oestrogen being assumed to contribute to variations in all physiology of behaviour; thus any studies in female animals require study across the oestrus cycle or at the same stage. However, this is not true. Studies that looked at numerous variables in both sexes found no differences, and this was independent of the stage of the oestrus cycle [75]. Hormone levels do fluctuate in female mice; however, hormone levels also fluctuate in male mice. The fluctuation is no more significant than in males, it is simply over a different time period [86]. A neuroscience meta-analysis on rats found no difference in trait variability between males and females [87], no evidence to suggest female rats produce more variable data at any stage of the oestrus cycle, and even when there were sex differences in the neurobiology there was no significant differences in the data variability. Therefore, if females do not produce more variable data there is no need to exclude them from studies. In the *Cln8*^{mnd} study the female mutant and control mice were analysed at all phases of their oestrus cycle with no differences found [27].

6.2. Cost

It is a common assumption that to include female mice requires using double the number of animals to ensure reliability, thereby increasing the financial cost of research [86] and an important ethical consideration in terms of animal numbers [88].

However, a well-designed factorial experiment can evaluate effects of treatment with essentially the same statistical power as a pair-wise test without doubling the sample size [86]. The only exception where a notable reduction in the statistical power occurs is when there is a significant difference in how males and females are responding to treatment. Scientifically, it would be biologically meaningful to design follow up testing and methodologies to explore sex differences.

Further, the cheapest part of the pipeline of therapeutic development is the preclinical stage; thoroughly exploring potential sex differences in animal models can save money in the long term, as this will lead to better designed and more cost-effective clinical trials, avoiding inefficacy or detrimental side effects in one sex that are not revealed until this stage or later.

6.3. Patriarchy

Discussion of why female animal models are not included in research must acknowledge an underlying misogyny in society. This dates back to the early 19th century [89], with the belief that the male brain is straightforward, rational and easy to study whilst the female brain is hormonal, irrational and difficult to study. The idea that women were intellectually inferior to men fell out of favour with most scientists post-Enlightenment, but was replaced by the theory that females are complementary to men; so where women are disordered and emotional, men provide stability. The supposed instability of women was then, and often still is, attributed to the menstrual cycle. The exclusion of female model organisms from research may stem from this misguided sexism with the belief that males are easier to study and even that males conduct more rationale research.

Certainly, research is dominated by men. Only an estimated 28 % of researchers worldwide are women; women scientists face a substantial gender pay gap [90]; and women scientists experience sexual harassment in the work-place (58 % of women in academia, according to a recent report from the USA [91]). If women scientists are not included

and valued as conductors of research in the laboratory setting, it is perhaps not surprising that the same applies to female model organisms.

Finally, there is a concern that repeated reporting of sex differences between males and females, particularly in terms of the brain and behaviour, may itself lead to increased sexism [89], with extrapolation of brain sex differences in model organisms to humans and perpetuating an idea that the brain of one sex may be "better" than the other. To mitigate this requires full results to be reported and explained accurately and clearly; there is nothing fundamentally sexist about acknowledging difference in neurobiology in brains from different sexes.

7. Strategies to remove sex bias and increase the use of female subjects

To address the major knowledge gap, females have had to be included as participants in clinical research studies since 1993 [92], and the inclusion of females and males is now approaching parity for that funded by USA National Institutes of Health (NIH) [93]. The increasing awareness of the importance of representation has led to recent measures by funding bodies encouraging the use of female animal models in basic science or preclinical research [94]. These include the European Commission [95], the Canadian Institutes of Health Research [96], and the USA NIH. Since 2016 NIH has implemented its Sex as a Biological Variable (SABV) policy for all funded research [72]. This encourages researchers to factor sex into the design, analysis and reporting of all vertebrate animal and human studies; both sexes must be included unless "strong justification" can be provided as to why only one sex is being studied, and if only one sex is used this should be stated in the title of the research. Analysing males and females together results in clustering of either sex around their individual means which not only obscures data on sexual dimorphism but weakens data as a whole [75]. Anticipated advantages in changing research design and analysing males and females separately includes the uncovering of sex differences prompting study of the basis for this. A review of past research in the field concluded that SABV would strengthen neuroscience research [97]. Surveying the response to this NIH policy shows that attitudes and grants including SABV are improving [88]. This NIH policy appears to have directly affected NCL research, since there has been an increase each year in considering SABV in NCL models since 2016 (Fig. 6).

Other national major funding bodies and editorial policies are expanding their requirements for including sex-specific reporting in research [71]. The European Commission for its new funding period 'Horizon Europe' is mainstreaming gender equality into all aspects of the application and award process, from research design to participation [98], as are national bodies such as UK Research and Innovation [99]. Further, a deliberate 'Gendered Innovations' approach can be taken to employ methods of sex, gender, and intersectional analysis to create new knowledge, with the aim to harness the creative power of this type of analysis for innovation and discovery in the field of science, health and medicine, engineering, and environment [100]. The impact of their interventions on the reporting of sex and female animal model representation should be monitored by funders.

A review of funders of NCL research in 2013 found 193 funding sources mentioned with the most frequently acknowledged the NIH (in 109 of the 295 (37 %) papers reviewed) [101], followed by two patient-led organisations (USA Batten Disease Support and Research Association and UK Batten Disease Family Association) and a major UK-based global charitable funder (The Wellcome Trust). These latter three do not currently have policies or guidance in place regarding the inclusion of both male and female model organisms. Affected families play a huge role in the research of rare diseases such as Batten disease. They provide valuable medical histories for research and raise funds for non-profit organisations, increase awareness, advocate for national funding, and motivate researchers. A further way to ensure the inclusion of SABV in Batten disease research is through families understanding its importance and putting pressure on researchers to adequately study sex differences.

It is well recognised that there are differences between males and females in terms of decision-making, collaboration, conflict resolution, and communication [102] which all contribute to experimental design, research implementation and interpretation. A more equal gender ratio of scientists would benefit the inclusion of model organisms of both sexes. A survey into the opinions of researchers on the 2016 NIH SABV found women scientists were significantly more likely than men to be in support of the SABV policy and more women than men believed it would increase reproducibility and rigour of research [88]; further, women scientists are more likely to conduct and publish research of relevance to women's health [103,104] which would automatically increase female model organism representation. Journals may increasingly require experimental design and analyses in their publications includes both sexes [105].

Much basic and disease focused research uses cells derived from patients, animal models and engineered cell lines, some of which are obtained from commercial providers. There is little identification or consideration of the sex of the organism from which these cells were derived [70,71], and this needs to be improved.

Finally, this topic is particularly timely given recent advances in gene editing technologies that can be used to produce litters of a single sex in laboratory mice and other engineered animal models [106]. Without better communication and understanding of the importance of considering SABV when using mouse models there could be further increase in this male bias if the production of male-only litters is adopted even when study of a single sex cohort is not justified by the underlying scientific question.

8. Conclusions

There are known sex differences in human CLN3 disease which accounts for nearly half of all NCL cases. There are no studies reporting whether sex differences occur in patients with other NCL types. However, there are sex differences reported in at least three mouse models of NCL diseases which suggests this should be investigated. With the ongoing focus on therapeutic development in NCL research it is important that any sex differences in Batten disease are recognised and accounted for.

We looked in detail at published studies using all animal models and especially mouse models which make up the vast majority of species used in NCL research. For all models of NCL we found that sex is still not valued in studies which could result in development of inadequate treatment particularly for females. This undervaluation of sex differences manifests in the frequent failure to state the sex of model organisms used in NCL research and the consistent omission of females. These shortcomings are likely the result of outdated beliefs regarding oestrogen and sample size. This may link with the wider gender inequality that exists in society which is seen in the underrepresentation of female scientists. The implementation of requirements by some funding bodies is an important driver to improve the consideration of sex differences in research, however, this has not yet fully impacted published NCL research.

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Author contributions

AM and SM devised the study. AM collected the references, analysed and interpreted the data, wrote the original draft, and prepared all the figures and tables. SM supervised the work, reviewed the analysis and data, edited the writing, and prepared the graphical abstract.

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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.

Data availability

The data is provided as part of the manuscript files

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