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Spinal muscular atrophy: Broad disease spectrum and sex-specific phenotypes

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Abstract

Spinal muscular atrophy (SMA) is one of the major genetic disorders associated with infant mortality. More than 90% of cases of SMA result from deletions of or mutations in the *Survival Motor Neuron 1* (*SMN1*) gene. *SMN2*, a nearly identical copy of *SMN1*, does not compensate for the loss of *SMN1* due to predominant skipping of exon 7. The spectrum of SMA is broad, ranging from prenatal death to infant mortality to survival into adulthood. All tissues, including brain, spinal cord, bone, skeletal muscle, heart, lung, liver, pancreas, gastrointestinal tract, kidney, spleen, ovary and testis, are directly and/or indirectly affected in SMA. Accumulating evidence on impaired mitochondrial biogenesis and defects in X chromosome-linked modifying factors, coupled with the sexual dimorphic nature of many tissues, point to sex-specific vulnerabilities in SMA. Here we review the role of sex in the pathogenesis of SMA.

Keywords

Spinal Muscular Atrophy (SMA); Survival Motor Neuron (SMN); X chromosome; mitochondria; male infertility; Intronic splicing silencer N1 (ISS-N1)

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Authors' statement

Authors acknowledge that the work is original and is not under consideration for publication elsewhere.

Declarations and competing interests

ISS-N1 target (US patent # 7,838,657) mentioned in this review was discovered in the Singh lab at UMASS Medical School (Worcester, MA, USA). Inventors, including RNS and UMASS Medical School, are currently benefiting from licensing of ISS-N1 target to IONIS Pharmaceuticals/Biogen, which is marketing Spinraza™ (Nusinersen), the FDA-approved drug, based on ISS-N1 target. RNS is co-founder of RNACorrect, Inc., an Iowa-based small business engaged in research and development.

Declaration of competing interests

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.

1. Introduction

Spinal muscular atrophy (SMA) is the leading genetic disease of children and infants affecting one in ~8,000 to 10,000 live births [1,2]. In more than 95% of incidences, SMA is caused by low levels of Survival Motor Neuron (SMN) protein due to deletions of or mutations in the *SMN1* gene [3]. *SMN2*, a nearly identical copy of *SMN1* universally present in humans, fails to prevent SMA since one of the *SMN2* coding exons, namely exon 7, is predominantly skipped, resulting in the production of an unstable truncated protein SMNΔ7 [4]. The broad spectrum of SMA pathologies has been categorized into five types: 0, 1, 2, 3 and 4 [5]. The extremely severe type 0 is characterized by death at or shortly after birth [6]. Type 1 (also called Werdnig-Hoffmann disease) age of onset is at birth; type 1 patients are never able to sit or walk and generally succumb to death before their 2nd birthday [7]. In the case of type 2 (also called Dubowitz disease) patients show symptoms before 18 months; they survive beyond 2 years, they can sit but cannot walk [7]. Type 3 (also called Kugelberg-Welander disease) is manifested after18 months of age and patients can sit, walk and survive into adulthood [7,8]. Type 4, the mildest form of the disease, arises during early adulthood and patients may survive well into their fifties and sixties [9]. While degeneration of motor neurons and abnormalities of neuromuscular junctions are typical manifestations in severe SMA [10], in mild SMA impairments of peripheral tissues, such as muscle, as well as cardiac defects and male infertility problems can emerge prior to neuromuscular phenotypes [11]. Thus, these impairments could become the primary concerns in mild form of the disease. Of note, substantial heterogeneity exists between patients within each SMA type. Part of this heterogeneity could be attributed to the disease modifying factors, the numbers of which continues to grow. Additional heterogeneity could be due to the specific effects of epigenetic factors, the role of which remains largely unknown. Remaining heterogeneity could come from the sex-specific attributes that are the focus of this review.

Mouse models have been extensively employed to understand the pathogenesis of SMA [12-14]. Unlike humans, mice carry a single copy of the *Smn* gene termed *Smn1*. Consistent with the prenatal death in type 0 SMA, deletion of *Smn1* in mouse is embryonic lethal [15,16]. Introduction of human *SMN2* rescues *Smn1* knockout mouse from the embryonic lethality and leads to the phenotype resembling that of the severe SMA [16,17]. A high copy number of *SMN2* decreases disease severity in both humans and mice [17-20]. A growing number of proteins, including plastin (PLS3), neuritin 1 (NRN1), neurocalcin delta (NCALD), TIA1 cytotoxic granule associated RNA binding protein (TIA1), Ubiquitin Like Modifier Activating Enzyme 1 (UBA1), Ubiquitin Specific Peptidase 9 X-Linked (USP9X), Stathmin-1 (STMN1), Myostatin (MSTN) and ZPR1 zinc protein (ZPR1), have been suggested to modify SMA severity [21-30]. Several of these modifying factors, namely PLS3, USP9X and UBA1, are encoded by the genes located on the X chromosome; hence they have the intrinsic capability to affect SMA severity in a sex-specific manner [21,26,27].

SMN is implicated in multiple cellular processes, including DNA repair, transcription, pre-mRNA splicing, translation, stress-granule formation, macromolecular trafficking, cytoskeletal dynamics and cell signaling [31]. While most functions of SMN require its interactions with other proteins [31], there is also evidence to support a direct interaction of

SMN with cellular transcripts [32]. The *SMN* genes generate a diverse repertoire of transcripts, including multiple alternatively spliced mRNAs, circular RNAs and long non-coding antisense RNAs [33-42]. Generation of these transcripts is likely to further expand the functions of *SMN*. Studies conducted in SMA patients and mouse models underscore the cell autonomous role of SMN in all tissues, including brain, spinal cord, liver, lung, heart, skeletal muscles, ovary and testis [Table 1; 43-137]. The tissue-specific requirement for SMN appears to vary depending on the developmental stage and the expression of the disease modifying factors [138]. In line with the distinct cell-specific roles, SMN impacts the expression of different genes in different tissues [139-141]. While not yet fully acknowledged, cumulative evidence supports the effect of sex on SMN functions as well as on the pathogenesis of SMA. Several recent reviews underscore the role of sex in genetic and epigenetic regulation as well as energy metabolism in mammals [142-145]. Sex also impacts the outcome of therapeutic interventions in several illnesses, including neurodegenerative, metabolic, and cardiovascular disorders [146-148]. Here we review the current literature to glean the sex-specific impact of SMN reduction that causes the disease. This review is inspired by independent reports supporting the adverse impact of low SMN on various tissues of males with mild SMA. SMN is particularly essential for maintaining male reproductive health [11], which deserves attention considering that male fertility remains a major global concern [149]. The less severe phenotype of SMA in females could be attributed to multiple sex-specific variables, including X chromosome-linked modifying factors, mitochondria and the action of the sex hormones.

The purpose of this review is to provide an assessment of SMN-dependent effects within cohorts of males and females rather than merely describe the differences between cohorts of males and females. Given the prominent role of SMN in gene regulation, including expression of factors with potential to be “overrepresented” in females such as those expressed from the X-chromosome and the mitochondrial genome, we expect SMN-linked sex-specific differences at all developmental stages. Further, due to changes in hormonal levels and the cumulative impact of the modifying factors, we anticipate a widening gap in SMN-dependent effects within cohorts of post-puberty patients in each sex category. While heterogeneity of the SMA patient population combined with the lack of sex-specific data in most reported studies may not allow sweeping claims of the gender-related effects, recent reports are beginning to capture differences between males and females caused by the loss of SMN. This review is aimed at motivating future studies in which sex-specific distinctions, regardless of their magnitude, are accurately captured. Understanding the effect of gender in SMA is critical for not only uncovering novel SMN functions but also for deciphering the role of a growing number of disease modifying factors and developing more efficient SMA therapies.

2. Sex and the prevalence of SMA

Major human diseases including cardiovascular diseases, Alzheimer’s disease (AD), Parkinson’s disease (PD), and amyotrophic lateral sclerosis (ALS) show sex-specific bias in their prevalence [145,150-152]. While women are preferentially impacted by AD and other dementias [150], more men than women are affected by PD and ALS [151,152]. Further, women show more resistance to oxidative stress-associated diseases such as ischemic heart

disease and ischemic stroke [145]. However, when it comes to muscular dystrophies, no clear generalizations can be made with respect to sex-specific differences, with Duchenne muscular dystrophy (DMD) and its milder form, Becker muscular dystrophy (BMD), being somewhat an exception. Both dystrophies are X-linked conditions that usually affect males. It should be noted, however, that some female dystrophin mutation carriers do present similar symptoms, particularly cardiac phenotypes, albeit in milder form [153-156]. At the same time, while men suffering from limb girdle muscular dystrophies (LGMD) show higher muscle fiber atrophy [157], women are more susceptible to developing musculoskeletal disorders [158]. Furthermore, in Facioscapulohumeral muscular dystrophy (FSHD), an autosomal dominant neuromuscular disorder with extremely variable symptoms [159], less females are affected than males [160]. A recent literature survey suggests differential interaction between a number of muscle pathologies and biological sex [161]. For instance, males and females display differences in muscle physiology, such as muscle size, muscle fiber composition, anabolic and catabolic factors as well as differences in circulating hormones and in mitochondrial content, biogenesis and activity [161-163].

Despite the fact that severe SMA is more prevalent than all other types of SMA combined [1], limited attention has been given to the sex-specific impact/differences in this type of the disease. The sex-specific statistics available for mild SMA are somewhat different. For instance, a study published in 1995 indicated that in the case of mild SMA, more males than females were affected by the disease [7]. In particular, this study found that in females the onset of mild SMA was delayed by about three years as compared to males [7]. While the study mostly analyzed Caucasian patients, findings are relevant to other ethnicities. For example, a recent study conducted on Japanese patients found a higher proportion of male cases of mild SMA [164]. Furthermore, an insurance claim study that analyzed the data from January 1, 2008 to October 1, 2015 found that adult patients with initial male infertility concerns were later diagnosed with mild SMA [11]. Of note, roughly equal number of males and females were represented in this insurance claim study [11]. Recently published results of the GTEx (Genotype-Tissue Expression) project, which investigated the impact of sex on gene expression among 44 different human tissues, found that ~37% of all genes show sex-biased expression [165]. Thus, strong evidence of the influence of sex on gene expression speaks to the SMA phenotype exhibiting sex-specific characteristics.

3. Role of mitochondria in sex-specific differences in SMA phenotype

Mitochondria play an important role in creating sex-specific pathologies including metabolic diseases, cardiovascular diseases, muscular dystrophies and neurodegenerative diseases [144]. The sex-specific effect of mitochondria is exerted through the activity of estrogens and estrogen receptors coupled with the asymmetric inheritance of the mitochondrial genome and the X chromosome. Throughout the evolution, mitochondrial DNA and X chromosomes spent more time under selective pressure in females than in males. Hence, genes located within mitochondrial DNA and on X chromosomes are better optimized to work in females than in males [166]. Mitochondria also impact the crosstalk between sex chromosomes and autosomes [167]. Studies in rodents and humans confirm sexual dimorphism in mitochondrial functions for multiple tissues including adipose tissue, skeletal muscle, cardiomyocytes and the brain. For example, in white and brown adipose tissue as

well as in skeletal muscle of rodents, females possess more functional mitochondria than males [168-170]. In humans, female adipose tissue has higher expression of genes involved in mitochondrial function than male adipose tissue [171]. In rodent cardiomyocytes, mitochondria are more efficient in females than in males [172].

Impaired mitochondrial biogenesis has been recorded in muscle of SMA patients as well as in motor neurons of SMA mice [82,173]. Interestingly, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), one of the key factors involved in the biogenesis of mitochondria, is downregulated in skeletal muscle of SMA patients and in SMA mice (Fig. 1) [82,174]. Numerous signaling pathways central to the adaptation of muscle to endurance training converge at PGC-1α [175]. Furthermore, expression of the transcription factor A, mitochondrial (TFAM) as well as the nuclear respiratory factors, NRF1 and NRF2, is downregulated in muscle of SMA patients (Fig. 1) [82]. Relevant to sex-specific effects, mitochondria regulate the biosynthesis of sex hormones (estrogen, progesterone and testosterone), which in turn regulate mitochondrial function [167]. Mitochondria are the major source of the energy supply. They also modulate many cellular processes including calcium and ionic homeostasis, reactive oxygen species generation, pH maintenance, steroid hormone production, lipid and carbohydrate metabolism and cell death. Levels of multiple respiratory chain subunits associated with the mitochondrial energy production are substantially downregulated in the skeletal muscle of SMA patients [82]. Hence, functional defects associated with the mitochondrial respiratory chain subunits could be also expected in other high-energy-consuming tissues, such as cardiomyocytes, neurons, liver and kidney, all of which are impacted in SMA (Table 1). A recent study conducted on a mild SMA mouse model captured sex-specific differences, as females displayed better endurance than males in the rotarod performance test [176]. The authors attributed these differences to better motor neuron function in females. Another study conducted using the mouse brain showed lower oxidative stress in mitochondria of females as compared to males [177]. Hence, it is likely that the cumulative effect of oxidative stress brings an additional sex-specific difference during the lifespan of SMA patients.

4. Sex-specific effect of X chromosome inactivation

In order to maintain appropriate dosage of X-chromosomal genes between males and females, one of the two female X chromosomes undergoes random and permanent transcriptional inactivation called X-inactivation or Xi. However, several X-linked genes, including those involved in protection against oxidative stress and proteolytic stress, escape inactivation, creating a dosage imbalance between the two sexes [145]. Three X-linked genes, *PLS3, USP9X* and *UBA1*, have been suggested to be positive modifiers of SMA [21,26,27]. *USP9X* codes for a deubiquitinase (USP9X), which deubiquitinates SMN and consequently stabilizes it against degradation by the proteasome machinery [26]. *UBA1* codes for Ubiquitin-like modifier activating enzyme 1 (UBA1), which directly interacts with SMN and mediates ubiquitin homeostasis and subsequent β-catenin signaling [178]. It has been shown that low levels of SMN cause downregulation of UBA1, leading to neuromuscular pathology due to disruption of ubiquitin homeostasis and β-catenin accumulation [178,179]. Systemic restoration of UBA1 ameliorates SMA phenotype in a mouse model of the disease [27]. The expression of both *USP9X* and *UBA1* is female-

biased due to Xi escape [145]. *PLS3* codes for an actin-binding protein, PLS3, which is another positive modifier of SMA [21]. It should be noted that the correlation between PLS3 expression and SMA severity is not absolute as other disease modifying factors may confound the effect of PLS3 [22,180]. Although *PLS3* is located within the X-chromosome evolutionary strata with the least chance of X-inactivation (Xi) escape, a published report showed that the gene was able to escape Xi in 4 out of 9 cases [181]. It also suggested that females would be variable in regard to PLS1 expression [181]. According to the same study, USP9X and UBA1 escaped from Xi completely [181]. Similarly, expression of UBA1 from the inactive X chromosome was demonstrated using female fibroblast cell line WI-38 [182]. One recent report found UBA1 to be expressed at higher levels in female cells as compared to male cells of dizygotic twins [183]. USP9X happens to be one of the six X-linked homologue of Y-linked genes. Interestingly, expression of USP9X has been shown to be significantly higher in female mouse brain as compared to male brain irrespective of its Xi status, potentially inducing sex-specific differences in neuronal function [184]. This finding supports a point of view that high expression of an X-inked gene could be achieved without Xi escape.

Other X-linked genes that may have sex-specific effects on SMA phenotype include the androgen receptor gene, cell-signaling receptor genes and microRNA genes [185]. In particular, location of the androgen receptor gene on the X-chromosome is suggestive of the impact this chromosome has on controlling the functions of the male sex hormone, testosterone. Furthermore, several X-linked genes are associated with enhanced female immunity against infections and with sexual dimorphism of the immune inflammatory response [185,186]. Impaired immune system and neuroinflammation are accompanying pathologies in SMA [126,128,187-189]. Future studies will determine if female SMA patients display milder inflammatory responses. About 10% of human microRNA genes are located on the X-chromosome and have the potential to exert substantial sex-specific effects on RNA metabolism in all tissues [190]. One of the X-linked microRNAs, miR-92a-2-5p, is predicted to bind circular RNAs generated by human *SMN* genes (Fig. 2) [41]. The role of miR-92a-2-5p has recently been implicated in mitochondrial translation [191]. Therefore, it is tempting to hypothesize that the sexual dimorphism in SMA could be partly regulated by X-linked microRNAs such as miR-92a-2-5p, availability of which is predicted to be modulated by *SMN* circular RNAs.

5. Importance of sex hormones

Differential exposure to sex hormones throughout embryonic development and adulthood is one of the driving forces behind sex-specific differences in the transcriptome and proteome of different mammalian tissues. Ovaries are the major source of estrogens, including 17β-Estradiol (E2), which plays an important role in both genomic (classical or canonical) and non-genomic (non-classical or non-canonical) regulatory mechanisms (Fig. 3). While the genomic mechanism refers to regulation of gene expression through chromatin remodeling and transcription, the non-genomic mechanism refers to regulation through posttranslational modifications, including phosphorylation of key intermediaries of signal transduction pathways. Among a large number of receptors that interact with E2, ERα plays an important role in cellular metabolism. ERα is considered to be a master regulator of transcription and

the key factor of sexual differentiation in the rodent brain [143]. For the purposes of clarity, we use ER and ERα interchangeably hereafter. ERα encompasses nuclear localization signal (NLS), ligand binding domain (LBD) and the DNA binding domain (DBD) (Fig. 3A). Binding of E2 to LBD triggers a structural change favoring a stable ER dimerization, co-regulator interactions and nuclear localization. Once in the nucleus, ERs regulate transcription (genomic regulatory mechanism) by directly interacting with the estrogen response elements (EREs) through their DBD (Fig. 3B). Alternatively, ERs can regulate transcription through interactions with other transcription factors, such as stimulatory protein 1 (SP1) and activator protein 1 (AP1) that are bound to their cognate recognition sites on DNA [192,193] (Fig. 3B). E2 and ERα play an important role in neuroprotection [194]. Interestingly, ERα also interacts with PGC-1α to upregulate transcription of NRFs that are associated with biogenesis of mitochondria [144]. In addition, ERs localize to the mitochondrion and regulate mitochondrial transcription (Fig. 1) [195]. Independent of transcription, ligand-bound ERs can regulate signaling pathways, including ERK/MAPK and PI3K/AKT signaling cascades (non-genomic regulatory mechanism), modulating cellular metabolism.

The testes are the primary source of testosterone, which mediates its functions through the androgen receptor (AR). AR encompasses the same domain structures as ER (Fig. 3A). Similar to ER, AR acts through both genomic and non-genomic regulatory mechanisms [196]. At birth, male mice are exposed to exceptionally high levels of testosterone, which is converted into estradiol (E2) in the brain leading to the male-specific changes of transcription through ER receptors [197]. These changes, coupled with the relative levels of sex hormones, contribute to dimorphic immune response in mammals [185,186]. The compromised immune response in SMA is likely to add to sex hormone-dependent dimorphism. The *SMN* promoter encompasses motifs of transcription factors such as SP1, ELK1 and CREB that are targets of the genomic and non-genomic regulatory pathways of ERα/ERβ and AR [38,198]. Hence, the relative expression of sex hormones could potentially bring sex-specific bias in the regulation of *SMN* transcription at least in some tissues.

6. Sex and cell-signaling pathways

Factors linked to cell-signaling pathways elevate levels of SMN [199]. For example, prolactin (PRL) and human growth hormone (GH) enhance *SMN2* transcription by activating the JAK/STAT pathway (Fig. 4) [200,201]. PRL and GH act through their cognate cell membrane receptors PRLR and GHR, respectively. PRLR and GHR share structural homology, including a cytoplasmic domain that provides a docking site for STAT5 [202]. It has been shown that STAT5 expression is affected by sex and that *STAT5* knockout mice show sex-dependent pathologies of peripheral tissues [203,204]. A recent study uncovered a female-specific bias in the mechanism of prolactin action as well as its localization in the mouse brain [205]. The canonical mechanism of PRL/GR action involves binding to their cognate receptors with subsequent STAT5 phosphorylation catalyzed by cytosolic tyrosine kinase, Janus kinase 2 (JAK2). Phosphorylated STAT5 is then dimerized and translocated to the nucleus where it regulates the expression of its target genes [202]. PRL/GR also participates in other signaling pathways including mitogen-activated protein kinase

(MAPK), AKT1/mTOR and NF-κB pathways that are known modulators of *SMN2* transcription and/or *SMN2* translation (Fig. 4) [174,206-209].

Insulin-like growth factor-1 (IGF-1) is a signaling molecule that serves as a neuroprotective factor in mammals (Fig. 4) [210]. While the level of IGF-1 in circulation is markedly reduced in SMA, expression of IGF-1 receptor (IGF-1R) is found to be increased in tissues of SMA patients as well as in mouse models of SMA [211-213]. SMA mice lacking one of the *Igf1r* alleles show extended life expectancy and improved motor behavior [213]. IGF-1R encoded by *Igf1r* directly affects PI3/AKT and MAPK/ERK pathways and impacts several other signaling pathways, including N-methyl-D-aspartate (NMDA) pathway, in an indirect way [210,214]. The sex-specific effect of IGF-1R depletion has been observed in several mouse models [215-217]. In particular, heterozygous female mice (harboring a single *Igf1r* allele) lived longer and showed better resistance to oxidative stress than males [215]. Future studies will reveal if IGF-R1 contributes towards SMA pathogenesis in a sex-specific manner.

7. Effect of sex on metabolic and tissue-specific defects in SMA

7.1. Effect of sex on metabolic defects

Metabolic defects in mammals impact multiple tissues, including liver, pancreas, gastrointestinal tract, kidney, reproductive organs and cardiovascular system. These tissues are likely to display sexual dimorphism due to disparities found between males and females in gene expression, immune response and xenobiotic metabolism as well as in sex hormone effects. SMA patients and SMA mouse models display abnormal glucose, lipid and amino acid metabolisms, although the magnitude of abnormalities differs with respect to the type of SMA and the patient's age [74,120,134,136,187,218,219]. Abnormal metabolism leads to reduced body weight in mouse models of SMA [12]. The reduction in body weight appears to be more prominent in females than males when compared to age- and sex-matched control littermates [13,119]. In other words, the effects of reduced SMN on body weight of female mice was greater than the one observed for male mice. These results may suggest that the loss of SMN differentially impacts the sex-specific network of interactions that account for the maintenance of body weight. In general, because of the extreme heterogeneity of SMA patients' phenotypes and a small cohort size employed in most studies, it is not feasible to capture sex-specific differences with high confidence. Yet, several of the metabolic disorders observed in SMA patients are known to have sex-specific bias. For instance, SMA patients have increased susceptibility to dyslipidemia, liver steatosis and non-alcoholic fatty acid disease (NAFLD) [74]. While NAFLD data for SMA patients were not analyzed to draw sex-specific inference [74], a recent report highlighted that the prevalence and severity of NAFLD were higher in men than in women of reproductive age [220]. Defective functioning of other tissues described below could also be due to aberrant metabolic processes with sex-specific impact in SMA.

7.2. Effect of sex on skeletal muscle defects

Multiple factors contribute towards sex-specific differences in the functionality of different muscle types [161]. Skeletal muscle is one of the first tissues to manifest structural and

functional defects in SMA (Table 1) [221,222]. While all types of skeletal muscles are affected by the disease [174], tibialis anterior (TA) muscle appears to be one of the most vulnerable [209]. Transcriptome and proteome analysis of muscles collected from an SMA mouse model revealed downregulation of PGC-1α, one of the key factors involved in biogenesis of mitochondria [174]. Among proteins secreted by muscle cells, C1q-TNF-related protein-3 (CTRP3) was shown to be downregulated in SMA [209]. This downregulation is consequential since CTRP3 was reported to promote *SMN* translation in motor neurons through activation of mTOR pathway [209]. Consistently, skeletal-muscle-specific depletion of SMN in mice leads to muscle fiber defects, neuromuscular junction abnormalities, compromised motor performance, and premature death [89]. Interestingly, female mice appear to be impacted more adversely than males [89]. These findings contrast a recent study showing higher susceptibility of skeletal muscle in males than females in a mild SMA mouse model [176]. The above discrepancy could be attributed to the fact that two different mouse models, one with selective depletion of SMN in skeletal muscle and the other with body-wide decrease in SMN levels, were used [89,176]. It is possible then that male mice are impacted more severely by body-wide depletion of SMN, while female mice are affected more when SMN depletion is restricted to skeletal muscle.

7.3. Effect of sex on neuromuscular junction and motor defects

Sex-specific effects on neuromuscular junction (NMJ) structure and motor functions in severe SMA are not easily captured as patients succumb to death before puberty. Recently, age and gender-specific motor defects were reported in a mild SMA mouse model [176]. In particular, male mice performed poorly on a rotarod test at 8-month compared to female mice of a similar age. The defects were attributed to the neurogenic atrophy and not to the loss of motor neurons. Analysis of TA muscle showed more atrophic fibers in males than in females [176]. An electrophysiological study performed on nerve-soleus muscle preparations of males showed less force generated upon stimulation of nerves than direct stimulation of muscle [176]. In contrast, a similar study on female samples showed no difference in force generated between nerve and muscle stimulation. These findings suggest possible disruption of signal transmission through NMJs in males but not in females. In addition, electrophysiological data pointed to possible deficiencies in NMJ/neuronal transmission in males as compared to females [176]. Blood circulation levels of CTRP3, which as described earlier promotes *SMN* translation, was shown to be higher in females than in males [209,223]. Thus, in addition to the protective effects against oxidative stress and favorable mitochondrial biogenesis in female neurons, high CTRP3 levels in blood may also benefit female neurons.

7.4. Sex and peripheral necrosis

Severe SMA patients, both males and females, occasionally display digital necrosis associated with thrombotic occlusions of small vessels [110,111]. However, due to a limited number of SMA patients with digital necrosis, no sex-specific inferences could be drawn. While human patients with mild SMA do not manifest digital necrosis, mouse models of mild SMA display tail and ear necrosis as hallmark features [13,16]. A sex-specific difference has been captured in the progression of peripheral necrosis in allele C mice that show mild SMA phenotype [224]. While the age of the peripheral necrosis onset was the

same in both sexes, male animals showed a faster progression of tail necrosis [224]. Autonomic nervous system abnormalities and vascular perfusion defects have been proposed as the potential causes of peripheral necrosis in SMA [110,111]. Interestingly, blood vessels were shown to manifest sexual dimorphism due to the role of ERα in flow-mediated remodeling of resistance arteries [225]. In addition, the production of reactive oxygen by mitochondria could impact vascular lesions in a sex-specific manner [226]. Future studies will determine if sex plays an important role in determining the severity of peripheral symptoms in SMA patients.

7.5. Effect of sex on pulmonary functions

Inadequate pulmonary function is one of the major causes of death in type 1 SMA patients [227]. Weakness of respiratory muscles coupled with respiratory tract infections also contribute towards the mortality associated with SMA. While sex-specific defects in pulmonary function have not yet been captured in type 1 SMA patients, the observational study published in 2011 indicated that among types 2 and 3 SMA patients, females may be associated with a greater decline in pulmonary function [228]. This could be due to female anatomical features such as small size of the rib cage and short diaphragm [229]. In addition, differential exposure to sex hormones and sensory feedbacks through vagal and spinal pathways could contribute to sex dimorphism in pulmonary function of SMA patients [229,230]. The severity of the disease is enhanced by hypoxia [231]. Age and sex differences in the ventilatory response to hypoxia have been reported in rats [232]. Hence, it is possible that the sex-specific defects in pulmonary function in SMA are in part due to the ventilatory response to hypoxia.

7.6. Effect of sex on renal injury

The kidney happens to be one of the affected organs in SMA [122,233,234]. Type 1 SMA patients display varying degrees of renal pathology such as tubular injuries, including loss of brush borders, flattened epithelium with detachment, and occasional protein casts [135]. Consistent with these findings, mouse models of severe SMA show developmental defects of kidney and substantial reduction in nephron counts [235]. Recently, a type 3 SMA patient was found to have mild renal dysfunction and proteinuria [234]. Intriguingly, patients suffering from acute kidney injury (AKI) show reduction of SMN levels in renal tubular cells, suggesting a crosstalk between kidney function and SMN [233]. Compared to wild type mice, heterozygous mice with a single *Smn* allele showed declined renal function and tubular injury upon ischemia-reperfusion injury (IRI) [233]. Of note, IRI-induced AKI is a significant clinical problem associated with prolonged hospital stay and morbidity [236]. Kidneys are known to have sexual dimorphism with regard to their anatomy and physiology [237,238]. For example, a study conducted on rats found higher renovascular resistance, lower absolute glomerular filtration rate (GFR) and lower renal plasma flow in females than males [239]. Men are known to have a higher risk of AKI at all levels of estimated GFR (eGFR) and albumin-to-creatinine ratio [238], although this observation has not been tested in SMA yet. The male-specific vulnerability to AKI has been linked to enhanced apoptosis in renal tubular cells triggered by testosterone-induced upregulation of Fas and Fas ligands [240,241]. At the same time, the female-specific protection against AKI has been connected

to the estrogen-induced sustained activation of cell survival pathways, including the Akt pathway [241,242].

7.8. Defects of reproductive organs

Abnormal development of the male reproductive system is one of the most consequential outcomes of low SMN in SMA. Independent studies conducted on SMA patients recorded cryptorchidism, a condition in which the testis fails to descend into the scrotum [65,127]. In several instances, cryptorchidism served as a good predictive marker of male infertility [243]. Young SMA patients (types 1 and 2) are not ideally suited to examine the effect of low SMN on male fertility; however, in adult SMA patients, male reproductive system dysfunctions have been documented [11]. Consistent with this, mild SMA mice (allele C model) show widespread degeneration of seminiferous tubules, loss of post-meiotic cells, and a drastic reduction in male fertility [118]. In contrast, female reproductive organs of allele C mice appear normal, although the uterus/ovary mass was reduced [118]. A recent study conducted in a severe mouse model of SMA found abnormalities in both ovary and testis [119]. This study confirmed the critical role of SMN in the survival of male germ cells and the maintenance of spermatogonia.

Testis shows the highest SMN expression as compared to all other tissues examined, suggesting the need for a sustainably higher level of SMN for various aspects of testis development and function [118]. Interestingly, unlike in other tissues, *SMN2* exon 7 is predominantly included in the testis. It appears that the *SMN2* exon 7 splicing switch from predominant skipping to significant inclusion fulfills, at least in part, the need for high SMN expression in human testes [118]. Additional mechanisms such as enhanced transcription and translation may also contribute towards the high expression of SMN in mammalian testes. Several cis-elements and transacting factors, including hnRNP A1/A2, hnRNP C, hnRNP Q, Tra2-β1, SRp30cPSF, Sam68, SRSF1, TDP-43 and TIA1, have been implicated in regulation of *SMN* exon 7 splicing [198,244-246]. Among them, hnRNP Q and Tra2-β1 have been linked to the promotion of *SMN2* exon 7 inclusion in testes [247,248]. However, the mechanism of the testis-specific splicing switch of *SMN2* exon 7 is not yet fully understood. Uncovering it has broad implication for understanding the tissue-specific splicing regulation as well as for finding novel therapeutic targets for manipulating *SMN2* exon 7 splicing.

In addition to testosterone production, another main function of testes is to produce sperm. Multiple signaling pathways (in both somatic and germ cells) cooperate to regulate spermatogenesis. In fetal testes, TGFβ signaling through somatic cells plays an important role in the development of fetal testicular germ cells [249]. Proliferation of Sertoli cells that feed the germ cells is regulated by cAMP/PKA, ERK1/2, PI3K/Akt and mTORC1/p70SK6 signaling cascades [250]. Self-renewal of spermatogonial stem cells is controlled by glial cell line-derived neurotrophic factor, GDNF, and RET Tyrosine Kinase pathway [251]. In postnatal testes, the retinoic acid signaling pathway regulates the differentiation of spermatogonia and entry into meiosis [252]. The quality and quantity of spermatogenesis is controlled by the FSH signaling pathway, which acts through the FSH receptor located on Sertoli cells [253]. Finally, withdrawal of testosterone leads to an arrest of spermatogenesis

in the post meiotic haploid phase [254]. Studies conducted using muscles and neurons of mouse models of SMA revealed dysregulation of several signaling cascades, including the most critical cAMP/PKA, ERK1/2, PI3K/Akt and mTORC1/p70SK6 cascades [174,199,209]. Future studies will uncover if relevant signaling pathways are particularly vulnerable to low SMN in specific cell types found in testes. In addition, it remains to be seen whether the aberrant expression of sex hormones by the reproductive organs causes a sex-specific impact on the development and function of other organs and tissues in SMA.

8. Sex-specific outcomes of SMA therapy

Considering that the overwhelming majority of SMA patients carry *SMN2*, correction of *SMN2* exon 7 splicing emerged as the most attractive therapeutic option for the disease [255-258]. The discovery of intronic splicing silencer N1 (ISS-N1) revealed how an intronic site could be effectively targeted by an antisense oligonucleotide (ASO) to fully restore *SMN2* exon 7 inclusion [258-262]. A number of in vivo studies independently validated the therapeutic efficacy of an ISS-N1-targeting ASO and confirmed the highest increase in the life expectancy in severe SMA mouse models [263-266]. Nusinersen (Spinraza), an ISS-N1-targeting ASO, became the first FDA approved therapy for SMA [260,261]. Gene therapy is the second approved therapy for SMA [267]. Both nusinersen and gene therapy have shown laudable outcomes in preventing death and ameliorating symptoms of the disease in SMA patients [268-270]. Recently approved Evrysdi (risdiplam) is the first orally deliverable small molecule for SMA therapy [271]. Similar to nusinersen, risdiplam promotes *SMN2* exon 7 inclusion through targeting the sequence and/or structural elements at the 5′ splice site of exon 7 [258,272,273]. Additional orally deliverable small molecules currently in clinical trial for the treatment of SMA target either the *SMN2* gene or downstream events [5,258]. While both sexes are benefitting from the currently approved drugs, no sex-specific outcome measures have been reported from preclinical and clinical studies of these drugs. However, a study conducted with an ISS-N2-targeting ASO showed sex-specific response in a mild SMA mouse model [224,274]. Of note, both ISS-N1- and ISS-N2-targeting ASOs abrogate the inhibitory context that sequesters the 5′ splice site of *SMN2* exon 7 [232,258,260]. In particular, the study indicated that SMA female mice displayed better therapeutic outcomes than male mice upon treatment with ISS-N2-targeting ASO [224]. This finding is consistent with a recent report showing a better functional performance in a rotarod test by females than males of a mild SMA mouse model [176].

9. Conclusions

The housekeeping protein SMN is mechanistically linked to important functions in most cellular compartments, including mitochondria, which are known to have female sex-specific adaptations due to maternal inheritance. Reduced levels of SMN cause SMA characterized by a broad spectrum of pathologies (Table 1). SMA severity could be modified by multiple protein factors, some of which are encoded by genes located on the X chromosome. The development of ovaries and testes, organs that produce sex hormones (estrogen and testosterone), are differentially impacted by low levels of SMN. Given the important role of sex hormones in cellular metabolism coupled with the sex-specific bias brought in by mitochondria and X chromosomes, sex is likely to impact the structure and

function of tissues expressing low levels of SMN in humans and animals. Unfortunately, the majority of SMA studies to date, either on animal models or patients, have used males and females together and interchangeably, without reporting any sex-dependent effects on disease progression, pathophysiology or response to treatment. Considering varied requirements of SMN in tissues at different developmental stages, capturing sex-specific variations during the early stages of SMA pathogenesis would be extremely valuable. However, this would require an arduous task of tissue-specific analysis of anatomical, physiological and biochemical parameters of sex- and age-matched patients coming from a similar genetic background. Recent studies employing animal models of SMA reveal sex-specific differences that increase with age [176]. Higher male susceptibility to the cumulative effects of oxidative stress [145] could be responsible for this observation. Testes appear to be one of the most and the earliest affected organs in all types of SMA. The adverse effect on testicular development with even a slight reduction of SMN provides a strong indication of the sexually dimorphic nature of SMA. Hence, deciphering the SMN-linked chain of molecular events during the testicular development has broad implications for uncovering novel transcriptional and posttranscriptional regulatory mechanisms.

Evaluating the sex-specific effects in model organisms and humans is of utmost importance for developing effective therapies [275,276]. There is a general concern about the immune response against the viral vector used for gene therapy for SMA [277]. Similar concerns could be raised for the antisense treatment as well as other potential SMA therapies [257,258,278]. Considering sex is an important determinant of immune response, the availability of sex-specific outcome measures would allow appropriate amendments to the existing treatment regimens or call for an altogether alternative treatment option. Emerging new data supporting sex-specific effects in motor neurons and other tissues in SMA warrant renewed efforts to discover novel therapeutic avenues for ameliorating the sex-specific pathologies in SMA.

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Highlights:

1. Spinal muscular atrophy (SMA) is a disease of broad spectrum affecting infants, children and adults
2. Most tissues and organs are intrinsically (and independently) affected in SMA
3. Sex plays a role in determining the overall prevalence and severity of SMA
4. Male reproductive organ development and male fertility are adversely impacted in SMA

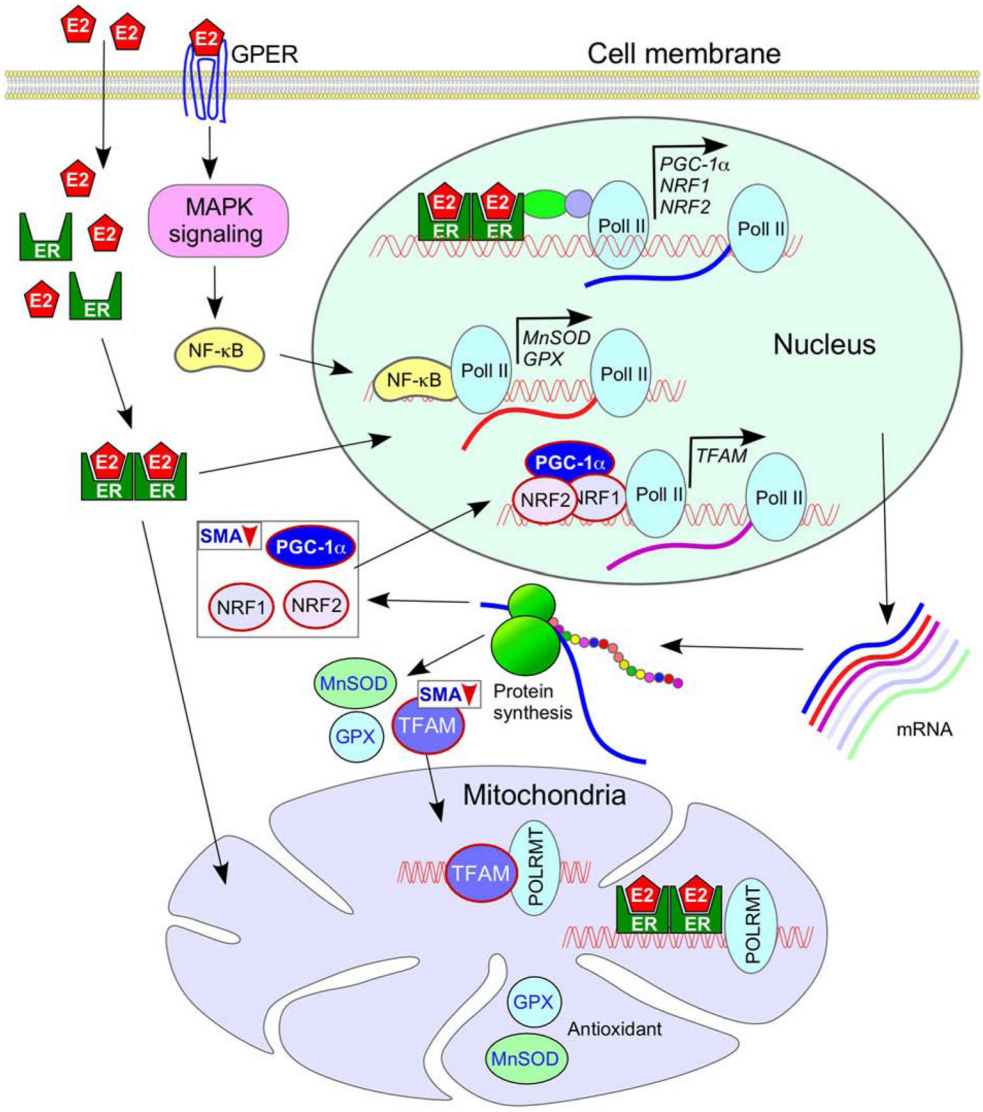

Figure 1.

Sex-specific effect of mitochondrial functions in tissues with high energy demand. Interactions of pol II and POLRMT with the nuclear and the mitochondrial DNA, respectively, have been shown. The female sex hormone estrogen (E2) regulates expression of transcription factors PGC-1α, NRF1 and NRF2 that are important for the expression of TFAM, a transcription factor. TFAM is essential for mitochondrial biogenesis. Both TFAM and E2 regulate expression of genes located on the mitochondrial genome. Through the MAPK signaling pathway, E2 also regulates expression of antioxidants GPX and MnSOD that localize to the mitochondria. PGC-1α, NRF1 and NRF2 are known to be downregulated in SMA, although it is not yet known if the downregulation is more pronounced in males. Details of pathways depicted here are described in the body of the main text and the accompanying references (see section 3). Abbreviations: E2, estrogen; ER, estrogen receptor; MnSOD, manganese superoxide dismutase; GPER, G protein-coupled estrogen receptor; GPX, glutathione peroxidase; MAPK, mitogen associated protein kinase; NF-κB,

nuclear factor kappa B; NRF, nuclear respiratory factor; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; pol II, RNA polymerase II; POLRMT, mitochondrial DNA-dependent RNA polymerase; TFAM, transcription factor A, mitochondrial.

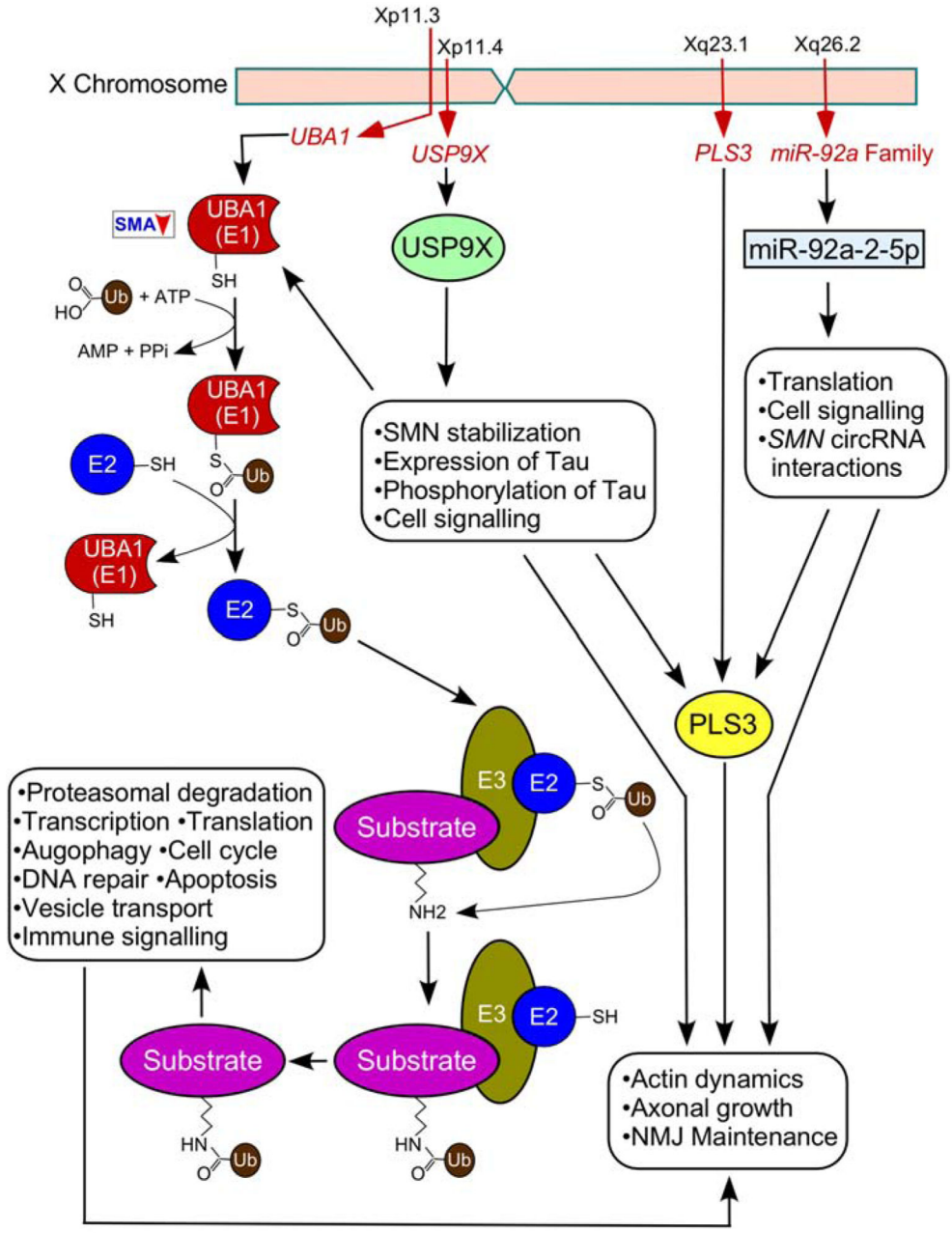

Figure 2.

Genes located on the X-chromosome modulate the severity of SMA. *USP9X* codes for USP9X, deubiquitinates SMN, protecting it from proteasomal degradation. *UBA1* codes for UBA1 that mediates ubiquitin homeostasis through direct interaction with SMN. *PLS3* codes for PLS3 and is a known positive modifier of SMA. PLS3 plays an important role in the maintenance of actin dynamics. X-linked coded miR-92a-2-5p is predicted to bind circular RNAs generated by the human *SMN* genes. Locations of the genes on the X-chromosome are indicated. Cellular functions of each protein and miR-92a-2-5p are listed in boxes. Details of pathways depicted here are described in the body of the main text and the accompanying references (see section 4).

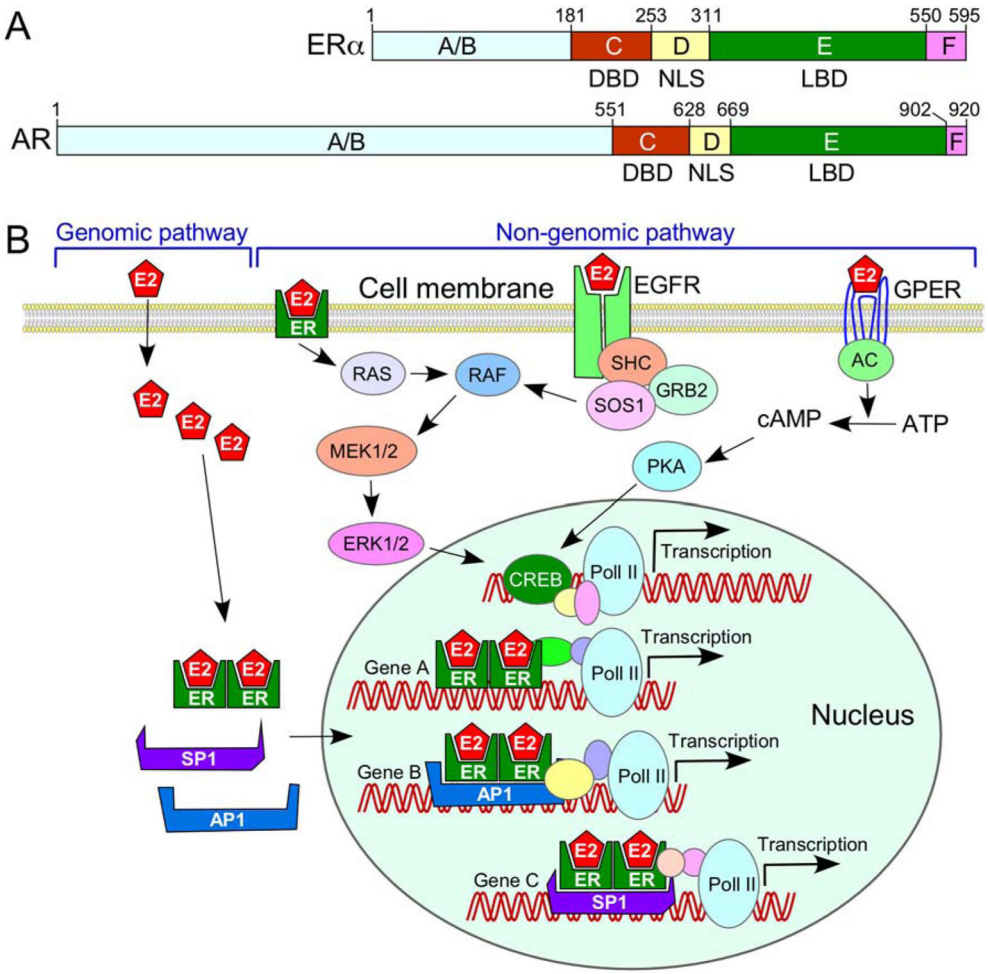

Figure 3.

Role of sex hormones and their receptors in the sex-specific effect on cellular metabolism. (A) Diagrammatic representation of the domain structure of estrogen (E2) receptor ER (ERα) and androgen receptor AR. Both ERα and AR contain A/B, C, D E and F motifs and possess a DNA binding domain (DBD), nuclear localization signal (NLS) and a ligand binding domain (LBD). (B) Diagrammatic representation of two E2-mediated pathways. The genomic pathway refers to the role of E2 in transcription regulation through direct interaction with DNA via ER and/or ER-associated transcription factors, such as AP1 and SP1. The non-genomic pathway refers to the role of E2 executed through signaling cascades initiated/triggered within the cytosol. Details of pathways depicted here are described in the body of the main text and the accompanying references (see section 5).

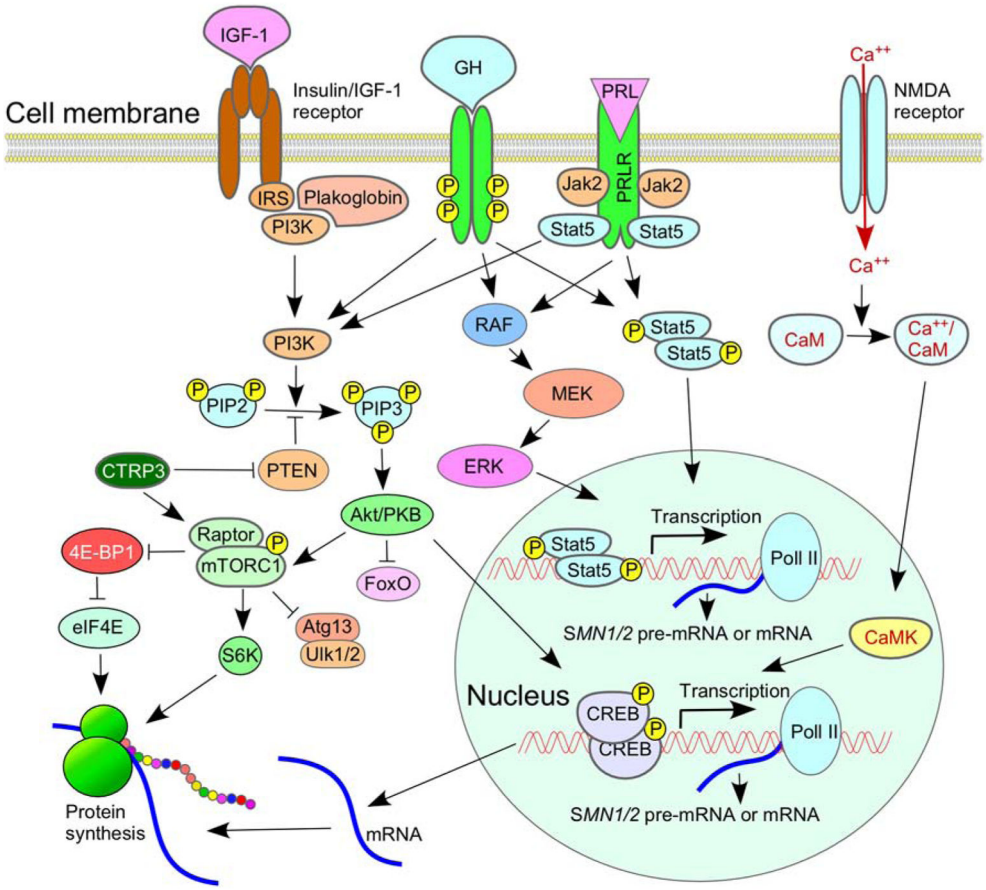

Figure 4.

Role of signaling pathways in SMA. Multiple signaling cascades are impacted by insulin-like growth factor 1 (IGF-1) and its receptor, both are aberrantly expressed in SMA. Prolactin (PRL) and human growth hormone (GH) enhance *SMN2* transcription by activating JAK/STAT pathway. Ca^{2+}/calmodulin-dependent protein kinase (CaMK) can also enhance *SMN2* transcription through recruitment of cyclic AMP response element binding protein (CREB) to the *SMN2* promoter. Details of pathways depicted here are described in the body of the main text and the accompanying references (see section 6).

Table 1.

Tissue-specific phenotypes of SMA.

Tissue/organ	Organism	Pathology/Symptoms	Refs.
Body composition	Human (T1,2)	Higher body fat mass, lower fat-free mass/lean body mass	43-45
Blood vessels	Mouse (SV)	Decreased capillary bed density in skeletal muscles, increased capillary calibre, reduced capillary ramification into muscle	46,47
Bone	Human (T1-3)	Low bone mineral density, congenital bone fractures, extremely thin ribs, generalized osteopenia, impaired cartilage formation	45,48-55
	Mouse (SV)	Impaired bone growth, pelvic bone fractures, decrease in total bone area, bone mineral content, bone mineral density	55,56
	Mouse (NSD)	Kyphosis of the spine	57
Brain	Human (T3)	Epilepsy, atrophy of parahippocampal gyrus	58
	Mouse (SV)	Impaired brain development	59
GI tract	Human (T2)	Gastroesophageal reflux, constipation, delayed gastric emptying	60,61
	Mouse (SV)	Morphological changes of intestine, increased inflamation, constipation, delayed colonic transit	61-63
Heart	Human (T0)	Congenital septal defect	64
	Human (T1-3)	Structural defects, bradycardia, ECG abnormalities, myocardial fibrosis	49,65-68
	Mouse (SV)	Structural defects, bradycardia, swollen and disorganized mitochondria, ECG abnormalities, blood clot accumulations	63,69-72
Lung	Human (T1-3)	Respiratory problems and need for ventillation	73
	Mouse (SV)	Pulmonary infarctions, lung emphysema, enlarged alveolar spaces	63
Liver	Mouse (SV,IN)	Abnormal liver development, atrophy, steatosis	72,74
	Mouse (LSD,EL)	Liver atrophy, severe liver dysfunction, iron overload, lack of regeneration	75
Skeleton	Human (T1-3)	Hip dislocation/subluxation, colapsing spine, scoliosis	11,65,76
	Mouse (SV)	Skeletal abnormalities in the lower body, reduced caudal vertebra	77
Skeletal Muscle	Human (T0)	Immature muscle fibers	78
	Human (T1-3)	Delayed muscle development, hypertrophy, impaired mitochondrial biogenesis, muscle weakness, lack of coordination	11,79-82
	Human (T4)	Abnormal gait	11
	Pig (NKD)	Muscle weakness, atrophy and fasciculations, decreased CMAP	83
	Mouse (SV,IN)	Morphological and functional deffects of muscle, impaired maturation of myofibers, macrophage depletion, muscle fatigue	16,63,84-88
	Mouse (MSD)	Severe degeneration, progressive myopathy, functional deficits	89-91
	Mouse (NSD)	Muscle atrophy, muscle fibre loss	57
	Drosophila	Reduced muscle growth, defective locomotion	92
Motor nerons	Mouse (SV)	Reduced axon growth, molecular and functional abnormalities	93,94
	Mouse (NSD)	Motor neuron degeneration	57
Central and peripheral nervous system	Human (T1,2)	Loss of motor neurons, abnormal sensory conduction, neuroinflamation	79,95-99
	Pig (NKD)	Loss of motor neurons	83
	Mouse (SV)	Myelination defects, sensory neuron developmental deffects, loss of synapse, functional deficits, neuroinflamation	100-106
	Mouse (NSD)	Reduction in lumbar and cervical motor neurons	57
	Zebrafish	Defects in motor neuron axonal outgrowth, abnormal branching	107,108

Tissue/organ	Organism	Pathology/Symptoms	Refs.
	Drosophila	Disfunctional sensory-motor network	92
Autonomic nervous system	Human (T1-3)	Sympathetic-vagal imbalance, changes in blood pressure, digital necrosis, sympathetic nerve hyperactivity	65,109-112
	Human (T2)	Gastroesophageal reflux and constipation	60
	Mouse (SV)	Decreased cardiac output, decreased contractility	69-71
	Mouse (SV)	Disrupted neuron signaling to colonic smooth muscles, diarrhea	61-63
NMJ	Human (T1)	Arrested/delayed NMJ development, functional abnormalities of NMJ	79
	Mouse (NSD)	Structural abnormalities of NMJs	57
	Mouse (SV,IN)	Structural and functionaly abnormalities of NMJs, decreased density and release of presynaptic vasicles	63,86,88,113-117
Central synapse	Mouse (SV)	Abnormalities in central synapse connectivity and function	101,102
	Mouse (NSD)	Defects in sensory-motor synapses	57
Ovary	Mouse (SV,MD)	Decreased weight of ovary comapred to body weight	118,119
Pancreas	Human (T1)	Abnormalities morphology of pancreatic islet cells, pancreatitis	65,120
	Mouse (Het)	Abnormalities of pancreatic islets	121
Kideny	Human (T1,2)	Impaired kidney structure and function, nephrocalcinosis	122,123
Spleen	Human (T1)	Abnormal development, abnormal function	124
	Mouse (SV,IN)	Reduced spleen weight, structural and functional defects	74,86,124-126
Testis	Human (T1,2)	Cryptorchidism	65,127
	Mouse (SV,MD)	Degeneration of seminiferous tubes, impaired spermatogenesis	118,119
Thymus	Mouse (SV,IN)	Lymphocite apoptosis, misregulated T-cell development	86,128
Urinary tract	Human (T1,2)	Urinary incontinence, enuresis, hypercalciuria	123,129
	Human (T3,4)	Nephrolithiasis,urinary retention problems	130
	Mouse (MD)	Reduced bladder and smooth muscle size	131
Vascular	Human (T1)	Digital necrosis, generalized inflammation of vasculature	110,111,132
	Mouse (SV)	Reduction vascular density in heart and small intestine	47,62,132
Metabolic	Human (T1-4)	Abnormal glucose and lipid metabolism, dicarboxylic aciduria, chronic hypercalcaemia, precocious pubarche, hyperlipoproteinemia	74,120,123, 133-137
	Mouse (IN)	Non-alcoholic fatty liver disease, hyperglycemia, hyperglucagonemia, increased insulin sensitivity, glucagon sensitivity	74,120
	Mouse (Het)	Abnormal glucose metabolsim, fasting hyperglycemia, hyperinsulinemia, hepatic glucagon sensitivity	121

Abbreviations: EL, late embryonic lethality; Het, heterologous for *Smn1* allele; GI, gastrointestinal; IN, intermediate phenotype; LSD, liver-specific depletion; MD, mild phenotype; MSD, muscle-specific depletion; NKD, motor-neuron-specific postnatal knockdown; NSD motor neuron progenitor-specific depletion; SV, severe phenotype; T0, SMA type 0; T1, SMA type 1; T2, SMA type 2; T3, SMA type 3; T4, SMA type 4;