Long non-coding RNAs in motor neuron development and disease

Vamshidhar R. Vangoor | Andreia Gomes-Duarte | R. Jeroen Pasterkamp

Department of Translational Neuroscience, University Medical Center Utrecht Brain Center, Utrecht University, Utrecht, The Netherlands

Correspondence
R. Jeroen Pasterkamp, Department of Translational Neuroscience, UMC Utrecht, Universiteitsweg 100, 3584 CG Utrecht, The Netherlands.
Email: r.j.pasterkamp@umcutrecht.nl

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Abstract

Long non-coding RNAs (lncRNAs) are RNAs that exceed 200 nucleotides in length and that are not translated into proteins. Thousands of lncRNAs have been identified with functions in processes such as transcription and translation regulation, RNA processing, and RNA and protein sponging. LncRNAs show prominent expression in the nervous system and have been implicated in neural development, function and disease. Recent work has begun to report on the expression and roles of lncRNAs in motor neurons (MNs). The cell bodies of MNs are located in cortex, brainstem or spinal cord and their axons project into the brainstem, spinal cord or towards peripheral muscles, thereby controlling important functions such as movement, breathing and swallowing. Degeneration of MNs is a pathological hallmark of diseases such as amyotrophic lateral sclerosis and spinal muscular atrophy. LncRNAs influence several aspects of MN development and disruptions in these lncRNA-mediated effects are proposed to contribute to the pathogenic mechanisms underlying MN diseases (MNDs). Accumulating evidence suggests that lncRNAs may comprise valuable therapeutic targets for different MNDs. In this review, we discuss the role of lncRNAs (including circular RNAs [circRNAs]) in the development of MNs, discuss how lncRNAs may contribute to MNDs and provide directions for future research.

Abbreviations: 5-FU, 5-fluorouracil; AAV, adeno-associated virus; ABA, Allen brain atlas; AD, Alzheimer’s disease; ADARB2, adenosine deaminase RNA-specific B2 (inactive); Ago2, Argonaute2; ALS, amyotrophic lateral sclerosis; arcRNAs, architectural RNAs; ASOs, antisense oligonucleotides; ATXN2, Ataxin-2; BDNF, brain-derived neurotrophic factor; bhHLH, basic helix-loop-helix; bpa, base pairs; C9-ALS, C9ORF72-associated ALS; C9ORF72, chromosome 9 ORF 72; ChIP, chromatin immunoprecipitation; circRNAs, circular RNAs; CLIP, cross-linking immunoprecipitation; CNS, central nervous system; Cpg, cytosine-phosphorus-guanine; DBPs, DNA-binding proteins; Dbr1, debranching enzyme 1; DE, differential expression; DOX, doxorubicin; DPR, dipeptide repeat; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; ESC, embryonic stem cells; FACS, fluorescence-activated cell sorting; FALS, familial ALS; FTD, frontotemporal dementia; FUS, fused in sarcoma; FUS/TLS, FUS/translocated in liposarcoma; GFP, green fluorescent protein; HCC, hepatocellular cancer; hESC, human embryonic stem cells; hiPSC, human-induced pluripotent stem cells; hpf, hours post-fertilization; hpsr, heat-shock RNA; HUVECs, human umbilical vein endothelial cells; IL, interleukins; INF, interferon; KCl, potassium chloride; LMC-MNs, limb innervating lateral motor column-motor neurons; lncRNAs, long non-coding RNAs; LPS, lipopolysacharide; MEFs, mouse embryonic fibroblasts; Malat1, metastasis-associated lung adenocarcinoma transcript 1; MATR3, Matrin 3; MeFS, mouse embryonic fibroblasts; Meg2, maternally expressed gene 2; mESC, mouse embryonic stem cells; Miat, myocardiac infarction-associated transcript; miRNAs, MicroRNAs; MND(s), motor neuron disease(s); MN(s), motor neuron(s); MN-TFs, MN transcription factors; MX1, Motor neuron and pancreas homeobox 1; NAC, nucleus accumbens; NAT, naturally occurring antisense transcript; NBs, nuclear bodies; ncRNA, non-coding RNA; NEAT1, nuclear-enriched abundant transcript 1; NONO, non-PoU domain-containing octamer-binding protein; Olig2, oligodendrocyte transcription factor 2; PAHs, polycyclic aromatic hydrocarbons; PBLCs, peripheral blood lymphocytes; PBMCs, peripheral blood mononuclear cells; PcG, polycomb group protein; PD, Parkinson’s disease; PIRNAS, Piwi-interacting RNAs; PLDs, prion-like domains; PRC1/2, polycomb repressive complex 1/2; PPSs, paraspeckle proteins; RA, retinoic acid; RAN, repeat-associated non-ATG-initiated translation; RBM14, RNA-binding motif protein 14; RBPs, RNA-binding proteins; Rmst, rhombomere somatogenesis 2-associated transcript; RNA-seq, RNA sequencing; rRNA, ribosomal RNA; SAL5, sporadic ALS; Sat III, satellite III repeat RNA; SCA2, spinocerebellar ataxia type 2; SCZ, schizophrenia; SFPQ, splicing factor proline/glutamine-rich; SINEs, short interspersed nuclear elements; SIRNAS, small interfering RNAs; Slc32a1, solute carrier family 32 member 1; SMA, spinal muscular atrophy; SN, substantia nigra; nscRNA, small non-coding RNA; SO, sense oligonucleotides; SOD1, superoxide dismutase 1; SSOs, splice-switching oligonucleotides; TARDBP, TAR DNA-binding protein; TDP-43, TAR DNA-binding protein 43; TLR, toll-like receptors; UTR, untranslated region; VAPB, vesicle-associated membrane protein-associated protein B/C; Xist, X-inactive-specific transcript.

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1 | INTRODUCTION

Over the past decade, the advent of high-throughput sequencing technologies coupled with advanced bioinformatics analysis has allowed an in-depth look into the non-coding part of the genome with exceptional resolution and scale. This has revolutionized our understanding of mammalian genome architecture, activity and regulation and led to the discovery that only $\pm 2\%$ of the human genome encodes proteins. This is remarkable given that the majority of nucleotides are transcribed, thus giving rise to an exquisitely high number of non-coding RNAs (ncRNAs) (Carninci et al., 2005; Djebali et al., 2012; Dunham et al., 2012). NcRNAs are subdivided on basis of their length into small ncRNAs ($<$200 nucleotides; e.g. microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs) and long ncRNAs (lncRNAs; $>$200 nucleotides), that differ in their biogenesis and function. LncRNAs show enormous functional diversity, influencing (post)-transcriptional and translational diversification of specific genes and gene networks (Qureshi & Mehler, 2012; Wang & Chang, 2011). It is estimated that approximately 2% of the human genome is transcribed into lncRNAs (Managadze et al., 2013; Palazzo & Lee, 2015). LncRNAs are mainly transcribed by RNA polymerase II (Guttman et al., 2009) and based on their location in the genome can be classified into the following five categories: (a) sense lncRNAs, transcripts transcribed from the sense strand of protein-coding genes and that contain one or more exons; (b) antisense lncRNAs, transcripts transcribed from the opposite strand of protein-coding genes and that contain one or more exons; (c) bidirectional lncRNAs, transcripts transcribed from the opposite strand that share the same promoter as another gene and that are usually located $<$1,000 base pairs (bp) away from each other in close genomic proximity; (d) intronic lncRNAs, transcripts transcribed from intronic regions without showing overlap with exons; and (e) intergenic lncRNAs, independent transcripts located in between two genes (Derrien et al., 2012; Ma et al., 2013). Another interesting class of lncRNAs are circular RNAs (circRNAs). CircRNAs are produced by back-splicing events that result in a closed loop structure, where a covalent bond is formed by linking 5’ (splice donor) and 3’ (splice acceptor) RNA splice sites. CircRNAs can be transcribed from both exonic and intronic regions, but the majority of circRNAs are exonic (Chen, 2016; Jeck et al., 2013; Memczak et al., 2013).

Mechanistically, lncRNAs can act at the transcriptional and post-transcriptional level to affect transcription, RNA processing and translation (Elling et al., 2016). Mediation of chromatin interactions, involvement in the formation of nuclear structures (e.g. paraspeckles), formation of decoys for transcription factors or as scaffolds for DNA-binding proteins and RNA-binding proteins (RBPs), functioning as miRNA sponges and control of mRNA decay are core functions of lncRNAs (Figure 1; Marchese et al., 2017; Quinn & Chang, 2016). Even though the majority of lncRNAs is present in the nucleus, some lncRNAs are found in the cytoplasm (Kung et al., 2013; Noh et al., 2018; Rinn & Chang, 2012; Ulitsky & Bartel, 2013). In the cytoplasm, lncRNAs are involved in processes such as RNA editing, RNA splicing and protein machinery assembly for translation initiation (Noh et al., 2018; Rashid et al., 2016). The majority of lncRNAs are of low abundance. In general, expression of lncRNAs is approximately 10-fold lower as compared to protein-coding transcripts (Cabili et al., 2011; Ravasi et al., 2006). This difference can in part be explained by prominent differences in lncRNA expression between cell types and tissues, and the strong spatiotemporal regulation of lncRNA expression as compared to mRNA expression (Cabili et al., 2011; Field et al., 2006).
et al., 2019; Yan et al., 2013; Yunusov et al., 2016). Ubiquitously expressed lncRNAs are generally expressed at high levels, while cell type- or tissue-specific lncRNAs, such as those in MNs, are often expressed at lower levels (Jiang, Li, et al., 2016). In addition to relatively low levels of expression, many lncRNAs show low levels of evolutionary and sequence conservation (Basu et al., 2013; Ulitsky et al., 2011). It has been suggested that evolutionary conserved lncRNAs may have important general functions, while species-specific lncRNAs could play relevant roles in biological processes that are crucial for a specific species (e.g. expansion of the brain in primates). Most of the lncRNAs discussed in this review are conserved among mammals (Table 1).

A remarkably large number of annotated lncRNAs, that is, approximately 40% (4,000 – 20,000 lncRNA genes), is expressed specifically in the brain (Derrien et al., 2012). In situ hybridization data from the Allen brain atlas show the expression of hundreds
of lncRNAs in specific regions of the mouse brain, in specific types of neural cells and even in specific subcellular compartments. Several of these expression patterns are similar to those described for coding mRNAs, hinting at important neuronal functions for lncRNAs (Mercer et al., 2008). Indeed, lncRNAs have been linked to processes such as neuron development, neurite growth, synaptic transmission, memory consolidation and ageing (Derrien et al., 2012; Mehler & Mattick, 2007; Mercer et al., 2008; Pereira Fernandes et al., 2018; Shi et al., 2017). In this review, we specifically focus on the proposed role of lncRNAs in motor neurons (MNs).

2 | LNCRNAS IN MOTOR NEURON DEVELOPMENT

2.1 | Linear LncRNAs in developing motor neurons

MN development is complex and depends on the interplay between different molecular factors. Several lines of experimental evidence link lncRNAs to the development of MNs (Figure 1a,b, see also Table 2).

2.2 | Meg3

The imprinted mammalian Dlk1-Dio3 locus produces multiple lncRNAs from the maternally inherited allele. Profiling of differentiating embryonic stem cell (ESC)-derived MNs revealed a predominant and gradual enrichment of maternally expressed gene 3 (Meg3) and other lncRNAs from the imprinted Dlk1-Dio3 locus (Rian and Mirtg) at post-mitotic stages of rostral MN development (Y. P. Yen et al., 2018). This enrichment was found not only in vitro in ESC-MNs but also in vivo in mouse spinal cord. Interestingly, both the over-expression of MN transcription factors and the treatment with retinoic acid

| Name                  | ncRNA          | Reported species | References                                           |
|-----------------------|----------------|------------------|------------------------------------------------------|
| Meg3, Rian and Mirtg  | IncRNA         | All mammals      | Ogata and Kagami, (2016) and Yen et al., (2018)      |
| CAT7                  | IncRNA         | All mammals      | Ray et al., (2016)                                   |
| Hoxb5os               | IncRNA         | Mouse and human  | Papaioannou et al., (2019)                           |
| Gm12688/ Gm14204      | IncRNA         | Mouse, human (NA)| Rizvi et al., (2017)                                 |
| LncMN–1,–2,–3 and Lhx1os | IncRNA     | Mouse and human  | Biscarini et al., (2018)                             |
| Lncrps25              | IncRNA         | Danio rerio and human | Gao et al., (2020) and Ulitsky et al., (2011) |
| Malat1                | IncRNA         | All mammals      | Ulitsky et al., (2011)                               |
| Rmst                  | IncRNA         | Birds and mammals| Chodroff et al., (2010)                              |
| Xist                  | IncRNA         | All mammals      | Johnsson et al., (2014)                              |
| Miat (Gomafu)         | IncRNA         | All mammals      | Chodroff et al., (2010); Sone et al., (2007)         |
| FUS-linked circRNAs   | circRNA        | Mouse and human  | Errichelli et al., (2017)                            |
| SMN circRNAs          | circRNA        | Mouse and human  | Ottesen et al., (2019)                               |
| NEAT1                 | IncRNA         | Mouse and human  | Clemson et al., (2009)                              |
| BDNFOS                | IncRNA         | Primates         | Lipovich et al., (2012)                              |
| TFEBα                 | IncRNA         | Human            | Davis et al., (2003)                                 |
| Myolinc               | IncRNA         | Mouse            | Militello et al., (2018)                             |
| SATIII                | IncRNA         | Drosophila melanogaster and human | Chung et al., (2018) |

Note: Overview of the reported conservation of lncRNAs discussed in this review. LncRNAs are listed according to their discussion in the main text.

Abbreviations: FUS, fused in sarcoma; Meg3, maternally expressed gene 3; NA, not annotated; SMN, survival motor neuron.
# Table 2: Overview of the expression and proposed function of lncRNAs in motor neuron development

| Name          | ncRNA Type | Regulation | Observation                                                                 | Mechanism                                                                 | References                  |
|---------------|------------|------------|------------------------------------------------------------------------------|----------------------------------------------------------------------------|-----------------------------|
| **Meg3**      | IncRNA     | Up-regulated; spatial regulation | Regulated throughout embryonic stem cells–motor neuron (ESC-MN) differentiation; enriched in the nucleus | Epigenetic regulation of Hoxa4:Hoxc5 expression                           | Yen et al., (2018)          |
| **CAT7**      | IncRNA     | Up-regulated | Regulated during early stages of human ESC-MN differentiation                 | Regulation of polycomb repressive complex 1 (PRC1) associated genes       | Ray et al., (2016)          |
| **Hoxb5os**   | IncRNA     | Up-regulated | Regulated throughout ESC-MN differentiation                                   | Tbd                                                                       | Rizvi et al., (2017)        |
| **Gm12688/Gm14204** | IncRNA | Cell type-specific expression | Uniquely expressed in V1/V1 and V2b GABAergic interneurons                   | Tbd                                                                       | Rizvi et al., (2017)        |
| **LncMN-1,-2,-3 and Lhx1os** | IncRNA | Cell type-specific expression; up-regulated | Specifically enriched in MNs; regulated during differentiation of mouse ESC (mESC)/ human-induced pluripotent stem cells (hiPSC)-derived MNs | Tbd                                                                       | Biscarini et al., (2017)   |
| **Lncrps25**  | IncRNA     | Down-regulated | Knockdown reduces swimming activity because of defects in primary MNs         | Via olig2 (Tbd)                                                          | Gao et al., (2020)          |
| **Malat1, Meg3, Rmst, Xist and Mlat** | IncRNA | Spatial distribution | Specifically enriched in somatodendritic/axonal fractions                  | Tbd                                                                       | Briese et al., (2016)       |
| **c-1, c-2, c-13, c-16, c-48, c-80, c-82, c-84, c-88** | circRNA | Up-regulated | Regulated during mESC/hiPSC-derived MN differentiation                      | Tbd                                                                       | Errichelli et al., (2017)  |
| **Human circSMN** | circRNA | Multiple isoforms produced | Primate specificity of SMN-derived circRNAs                                 | Tbd                                                                       | Ottesen et al., (2019)      |

Abbreviations: hiPSC, human-induced pluripotent stem cells; Meg3, maternally expressed gene 3; mESC, mouse embryonic stem cells; Tbd, to be determined.
in a normally maternally inherited methylated region context (IG-DMR\textsuperscript{mat} \textsuperscript{+} ESC induced Meg3 expression. Therefore, these factors, that are crucial for MN development, may drive Meg3 expression during differentiation of ESCs into rostral brachial MNs. Meg3 was found to be enriched in the nucleus of MNs while being chromatin-associated. This strongly suggested a role in gene regulation. Indeed, by combining gene knockdown and chromatin immunoprecipitation, Meg3 was shown to facilitate polycomb repressive complex 2 (PRC2)-Jarid2 interactions in post-mitotic MNs. Meg3 knockdown led to a global down-regulation of H3K27me3 and rostral Homeobox (Hox) genes, and an up-regulation of MN progenitor genes (Pax6 and Irx3) and of caudal Hox genes (a Hox5 to Hoxc8 shift). Hox genes are transcription factors that regulate MN fate along the rostro-caudal axis of the central nervous system (CNS). Based on these and other findings it was proposed that Meg3 facilitates PRC2-Jarid2 complex formation to perpetuate rostral MN cell fate by maintaining the silenced epigenetic state of MN progenitor and caudal Hox genes (Y. P. Yen et al., 2018).

### 2.3 | CAT7

Another screen designed to detect IncRNAs co-precipitating with polycomb repressive complex 1 (PRC1) from chromatin identified multiple IncRNAs that influence the expression of polycomb group protein-regulated genes. Loss of a novel IncRNA, CAT7, led to an increase in MN and pancreas homeobox 1 (MNX1 or HB9) expression in human embryonic stem cells-derived MNs (Ray et al., 2016). MNX1 is a transcription factor that regulates the formation of pancreatic beta islets and MNs (Bonnefond et al., 2013; Van Arensbergen et al., 2010). During MN development, MNX1 expression is silenced at early stages of differentiation as a result of PRC1 binding to the MNX1 locus (Van Arensbergen et al., 2010). CAT7 enhances the binding of PRC1 to the MNX1 locus before activation of pathways that determine MN fate. CAT7 shares high sequence similarity to a non-syntenic \textit{Danio rerio} (zebrafish) analog, zcat7l. Depletion of zcat7l by morpholino interference resulted in microcephaly at 48 hr post-fertilization (hpf) and mortality starting on day 5. These phenotypes were successfully reverted by co-expression of the human CAT7 IncRNA isoform, indicating possible conserved functions between these non-syntenic IncRNAs. The phenotypes found following zcat7l depletion were also observed following knockdown of the zebrafish polycomb complex proteins bmi1a/b, and zcat7l and bmi1a/b genetically interacted in combined knockdown experiments. Overall, these data indicate a functional link between CAT7/zcat7l IncRNAs and PRC1 in human and zebrafish. More generally, these findings show that IncRNAs can regulate and promote transcription activation at specific loci (Ray et al., 2016).

### 2.4 | Gm12688 and Gm14204

In another study, single-cell topological RNA sequencing analysis was used to characterize the mechanisms underlying in vitro differentiation of mouse embryonic stem cell (mESC)-derived MN cultures (Rizvi et al., 2017). Specific temporal regulation of IncRNAs was found during pluripotency, at the neural progenitor stage and during the transition to neuronal maturation. Different stages of MN differentiation are characterized by waves of Hox gene activation (Figure 1a). Intriguingly, these waves were accompanied by an up-regulation of IncRNAs that originate from the opposite strand of these genes. This co-expression is in line with previous reports identifying IncRNAs as regulators of Hox gene clusters (Dinger et al., 2008; Ponjavic et al., 2009; Rinn et al., 2007; Wang et al., 2011). In addition, several IncRNAs were detected in other neuron types in the cultures, which contain MNs and concomitant cell types (i.e. those found in the spinal cord). For example, Gm12688, an intergenic IncRNA located near Foxd3 and transcribed from the opposite strand, was found in V1 interneurons. Gm14204, an intergenic IncRNA located near \textit{Solute carrier family 32 member 1} (Slc32a1) and transcribed from the opposite strand, was restricted to V1 and V2b GABAergic interneurons (Rizvi et al., 2017). The discovery of these intergenic IncRNAs suggests that IncRNAs may play a role in neuronal diversification in the spinal cord.

### 2.5 | Lhx10s, Lnc-MN1, LncMN-2 and LncMN-3

Stage-specific expression of IncRNAs was also detected in an analysis of the long non-coding transcriptome of mESC- and human-induced pluripotent stem cells (hiPSC)-derived MNs purified by fluorescence-activated cell sorting (FACS). The expression of several IncRNAs was restricted to MNs, as compared to absence of signal in green fluorescent protein-negative cells (i.e. MN progenitors and interneurons). Interestingly, these IncRNAs showed a predominant cytoplasmic expression (Biscarini et al., 2018). Expression of a subset of mouse IncRNAs (Lhx10s, 2610316D01Rik, 5330434G04Rik and A730046J19Rik) was specifically increased at later stages of ESC-MN development (from day 4 onwards), during which MN progenitors develop into post-mitotic MNs and differentiate further. This IncRNA cluster was conserved in human (renamed Lhx10s, Lnc-MN1, LncMN-2 and LncMN-3) and showed a similar mode of expression regulation during hiPSC-derived MN development. These results further confirm the role of IncRNAs in MN differentiation and hint at strong evolutionary conservation of the mechanism-of-action and function of IncRNAs in MNs.

### 2.6 | Malat1, Meg3, Rmst, Xist and Miat

In addition to studies reporting general changes in IncRNA expression, other work has focused on the spatial enrichment of these non-coding RNAs in specific subcellular compartments. Initially, IncRNAs had predominantly been detected in the nucleus and were therefore studied for potential roles in the regulation of gene expression (Sun et al., 2018; Ulitsky & Bartel, 2013). The presence of IncRNAs in other subcellular compartments hinted at the exciting possibility...
of additional and different molecular functions. Briese et al. (2016) investigated the abundance of several lncRNAs in the axonal or somatodendritic compartment, including that of metastasis-associated lung adenocarcinoma transcript 1 (Malat1), Meg3, rhabdomyosarcoma 2-associated transcript (Rmst), X-inactive-specific transcript (Xist) and Myocardial infarction-associated transcript (Miat). While some lncRNAs showed similar levels in different compartments, Meg3 and Rmst were enriched in somatodendritic and axonal fractions, respectively (Briese et al., 2016). The molecular mechanisms dictating this highly specific distribution are unknown. However, a number of lncRNAs can interact with proteins and it is plausible that protein:RNA complexes play a role in compartment-specific lncRNA distribution (Briese et al., 2016; Quan et al., 2017).

2.7 | Lncrps25

While many of these studies reveal interesting lncRNA expression patterns that hint at roles during MN differentiation, recent work has also begun to explore the functional role of lncRNAs in MNs in vivo. For example, the lncRNA lncrps25 was found to be strongly expressed in the embryonic zebrafish CNS and co-expressed with mnx1, while lncrps25 knockdown induced body abnormalities and striking changes in locomotor behaviour, that is, decreased swimming distance and speed (Gao et al., 2020). These phenotypes were caused by defects in primary MNs that showed reduced axon length and branching. Lncrps25 depletion led to a reduced expression of oligodendrocyte transcription factor 2 (Olig2) in brain and spinal cord. Olig2 co-expression could partially rescue the motor phenotypes caused by lncrps25 depletion. Olig2 is part of the Olig gene family that encode for basic helix-loop-helix transcription factors that are required for MN and oligodendrocyte development (Lu et al., 2002; Park et al., 2002). Based on the effect of lncrps25 depletion, and lncrps25 and olig2 expression patterns, it was proposed that lncrps25:olig2 interactions occur at the neural plate stage to eventually affect MN development (Gao et al., 2020). However, the precise mechanistic details of this lncRNA–mRNA pathway remain to be defined. A recent study focusing on defining lncRNA networks in oligodendrocytes identified the oligodendrocyte-specific lncRNA LncOL1. LncOL1 is involved in oligodendrocyte differentiation and CNS remyelination (He et al., 2017). Interestingly, a peak in genomic occupancy by Olig2 was detected around the transcription start site of LncOL1, suggesting a role for Olig2 in the transcriptional regulation of LncOL1. Therefore, Olig2 appears to be a shared factor in the regulation of MN and oligodendrocyte differentiation through interactions with different lncRNAs (lncrps25 and LncOL1). This is intriguing since the specification of MN and oligodendrocytes from ventral progenitor cells is a sequential process mediated by olig2 (Ravanelli & Appel, 2015). Further, oligodendrocytes are known to play a role in MN excitability and death, and consequently to subsequent oligodendrocyte-related pathology (Ferraiuolo et al., 2016). It will be interesting to determine how Olig2:lncRNA networks interact during MN and oligodendrocyte development, and how their dysfunction may lead to disease.

2.8 | CircRNAs in developing motor neurons

CircRNAs are highly enriched in the brain, dynamically regulated and conserved across species (Rybak-Wolf et al., 2015). The mechanisms that cause brain-enriched circRNA expression remain to be determined. One explanation is that many circRNAs derive from genes that are enriched in brain tissue, but the existence of neuron-specific biogenesis mechanisms has also been proposed (van Rossum et al., 2016). Another interesting observation is that whereas linear lncRNAs often display cell type-specific expression (e.g. in MNs), cell type-specific circRNAs are rare. This difference may arise from the fact that whereas many lncRNAs originate from intergenic stretches of the genome that lack protein-coding genes, circRNAs are generally produced from protein-coding transcripts (Memczak et al., 2013; Ransohoff et al., 2018; Salzman et al., 2012). MN-specific circRNAs therefore mainly arise from MN-specific genes, while the intergenic regions that produce other lncRNAs may be susceptible to different kinds of (post)transcriptional regulation. CircRNAs are differentially expressed across brain regions, subcellular compartments and regulated at pre- and postnatal stages (Chen & Schuman, 2016; Memczak et al., 2013; Rybak-Wolf et al., 2015; Vena et al., 2015; You et al., 2015). Many of these RNA molecules derive from genes that encode for synaptic proteins or proteins with essential roles in neural development. Further, the expression of several circRNAs is influenced by neuronal activity and they are shown to accumulate during ageing (Vena et al., 2015; Westholm et al., 2014). Together, these observations hint at roles for circRNAs in a wide range of neuronal processes in brain development, function and disease (van Rossum et al., 2016).

The functional roles and mechanism-of-action of most circRNAs remain poorly understood. A detailed discussion of the function of circRNAs is beyond the scope of this review, but several other recent reviews cover our current knowledge of the molecular effects and functional roles of circRNAs (Ebbesen et al., 2016; Meng et al., 2019; van Rossum et al., 2016; Salzman, 2016; Sekar & Liang, 2019). Translation into small peptides, miRNA sponging and sequestration of RBPs are some of the currently accepted mechanisms by which circRNAs can act (Li et al., 2018).

2.9 | FUS-linked circRNAs

CircRNAs have also been detected in MNs. Hundreds of circRNAs were found in mESC-derived MNs and, similar to that has been demonstrated for other lncRNAs, their expression increased during MN differentiation (Errichelli et al., 2017). The majority of identified circRNAs was conserved in hiPSC-derived MNs. In addition to expression profiling, this study also provided insight into the mechanisms underlying circRNA biogenesis in MNs. The
ALS-associated protein fused in sarcoma (FUS) was shown to promote circRNA formation in MNs without affecting their linear counterparts. FUS is an RBP with roles in RNA splicing and may contribute to circRNA formation by regulating the back-splicing events that lead to RNA circularization. In line with this idea, cross-linking immunoprecipitation (CLIP) assays revealed a specific enrichment of FUS at circularizing exon–intron regions of selected circRNAs (c-1, c-2, c-13, c-16, c-48, c-80, c-82, c-84, c-88) (Errichelli et al., 2017; Verheijen & Pasterkamp, 2017).

2.10 | SMN circRNAs

Although the biogenesis and functions of circRNAs are often evolutionarily conserved, in some instances the abundance, the presence of specific isoforms or functions of circRNAs are species-specific (Rybak-Wolf et al., 2015). Here, it is worth mentioning the human survival motor neuron (SMN) locus, which is able to originate a surprisingly large number of different exonic circRNAs (Ottesen et al., 2019). SMN protein is essential for MN development and survival and its loss causes SMA (Chaytow et al., 2018; Fallini et al., 2012; Lefebvre et al., 1995). Most of the reported functions of SMN are linked to RNA processing. However, it is known that SMN protein accumulates in the axons of MNs during development hinting at roles in, for example, local gene expression regulation (Gabanella et al., 2005; Giavazzi et al., 2006; Groen et al., 2013; Zhang et al., 2006). In addition to SMN protein, the SMN locus also generates a vast array of circRNAs. Using intron–exon site complementarity analysis in MN-like NSC-34 cells (mouse MN-like hybrid cell line), the mouse Smn locus was predicted to give rise to a considerably lower number of circRNAs as compared to the human SMN locus (Ottesen et al., 2019). These differences seem to result from a lower number of short interspersed nuclear elements in the intronic regions of the mouse Smn gene. A substantial number of SMN-derived circRNAs was specific to primates. It is therefore possible that specific circRNAs may be directly linked to MN functions that have become more important during evolution. The consequence of the species-specific expression of SMN-derived circRNAs and the functional roles of these IncRNAs remain to be determined. Nevertheless, this insight highlights the need for more knowledge on how genetic differences influence non-coding RNA biogenesis and function.

3 | LncRNAs in Motor Neuron Disease

LncRNAs are strongly regulated by exposure to drugs, environmental factors (e.g. toxins) or as a consequence of pathological situations. In many instances, regulation of lncRNA expression confers neuroprotection or induces anti-apoptotic responses (Table 3). Given this regulation of lncRNAs, their dynamic expression patterns in MNs and their emerging roles in MN development and function, it is not surprising that lncRNA dysregulation has been implicated in MNDs. Understanding the mechanisms-of-action and functions of lncRNAs may assist the development of new therapies for MNDs. Here, we discuss and summarize the expression and functional role of lncRNAs in different MN-related diseases (see also Table 4), extending on previous reviews (Chen & Chen, 2020; Gagliardi, et al., 2018).

3.1 | Amyotrophic lateral sclerosis (ALS)

ALS is a fatal disease characterized by the progressive loss of MNs in brain and spinal cord. About 90%–95% of cases lack a clearly identifiable hereditary or environmental cause classically being referred to as sporadic ALS (sALS). About 5%–10% of cases are defined as familial ALS (fALS) because of demonstration of the direct inheritance (Renton et al., 2014), although the simple distinction between sALS and fALS is no longer tenable (Al-Chalabi et al., 2016). In most countries, the prevalence of ALS is approximately four to six cases per 100,000 people, with a median age of onset of 65 years for sALS, whereas this is 10 years earlier for fALS cases (Al-Chalabi & Hardiman, 2013; van Es et al., 2017). As disease progresses, a large proportion of upper (corticospinal) MNs, projecting from the motor cortex to the brainstem and spinal cord, and bulbar and spinal MNs, projecting to skeletal muscles, degenerate. This ultimately leads to spasticity and muscle atrophy which eventually results in weakness and paralysis. Eventually, patients die as a result of paralysis of respiratory muscles within 3–5 years after symptom onset (van Es et al., 2017).

The causative mechanisms that lead to MN degeneration and ALS remain incompletely understood. Mutations in more than 30 genes have been linked to ALS, and based on the functions of these genes, multiple disease pathways have been proposed and investigated. The most commonly mutated genes are chromosome 9 ORF 72 (C9ORF72; hexanucleotide (GGG GCC)_n (G,C) repeat expansion in a non-coding genomic region), superoxide dismutase1, TAR DNA-binding protein and FUS (Renton et al., 2014). These genes regulate molecular pathways controlling key cellular events such as RNA biology, protein turnover and axonal transport (Burk & Pasterkamp, 2019; Robberecht & Philips, 2013).

The RBPs TAR DNA-binding protein 43 (TDP-43) and FUS are normally located in the nucleus and influence RNA metabolism. In ALS MNs, an abnormal accumulation of these proteins is observed in the cytoplasm that is thought to contribute to MN degeneration because of effects on RNA processing and other RNA-related mechanisms (Blokhuys et al., 2013). Further, the ALS-associated hexanucleotide repeat expansion in the C9ORF72 locus causes mutant C9ORF72 pre-mRNA to accumulate in RNA foci leading to accumulation of RBPs (Gendron et al., 2013; Renton et al., 2014). Given these and other data supporting a prominent role for defects in RNA biology in ALS, it is not surprising that lncRNAs also contribute to the development of ALS and other MNDs. Here we discuss a few lncRNAs that have been implicated in the pathogenesis of ALS or SMA.
### TABLE 3  Expression changes in motor neuron lncRNAs in response to exposure to external factors or drugs

| LncRNA | Treatment/stimulus | Response | (Neuro) protection or Disease onset | System | References |
|--------|-------------------|----------|------------------------------------|--------|------------|
| Meg3   | 5-aza-2-deoxycytidine (demethylating agent) | Up-regulation | Tbd | Human breast cancer tumour cell lines; Human meningioma cell line; human hepatocellular cancer (HCC) cell lines | Braconi et al., (2011); Zhang et al., (2010) and Zhao et al., (2005) |
|        | Hypoxia           | Up-regulation | Tbd | Human umbilical vein endothelial cells (HUVECs) | Michalik et al., (2014) |
|        | Serum starvation  | Up-regulation | Tbd | Mouse embryonic fibroblasts (MEFs) | Wang, Liang, et al. (2019) |
|        | Heroin            | Up-regulation | Tbd | Human nucleus accumbens (NAC) | Michelhaugh et al., (2011) |
| Hoxb5os| Serum starvation  | Down-regulation | Tbd | MEFs | Wang, Liang, et al. (2019) |
| Malat1 | Polycyclic aromatic hydrocarbons (PAHs) exposure | Up-regulation | Tbd | Peripheral blood lymphocytes (PBLCs) of male coke oven workers | Gao et al., (2016) |
|        | Hypoxia           | Up-regulation | Tbd | HUVECs; Several tissues of mice exposed to hypoxia (including brain) | Lelli et al., (2015); Michalik et al., (2014) |
|        | X-irradiation     | Up-regulation | Tbd | Human lymphoblast cell line TK6 | Chaudhry, (2014) |
|        | β-Asarone         | Down-regulation | Neuroprotective | In vitro and in vivo (mice) | Zhang et al., (2016) |
|        | doxorubicin (DOX), 5-fluorouracil (5-FU), Mitomycin | Up-regulated | Anti-apoptotic | DOX, 5-FU and Mitomycin-resistant HCC cells | Yuan et al., (2016) and Zhang et al., (2020) |
|        | Heroin            | Up-regulation | Tbd | Human NAC | Michelhaugh et al., (2011) |
| Rmst   | X-irradiation     | Up-regulation | Tbd | Human lymphoblast cell line TK6 | Chaudhry, (2014) |
| Xist   | DOX               | Up-regulation | Tbd | DOX-resistant colorectal cancer tissues and cells | Zhu et al., (2018) |
| Miat   | (Gomafu)          | Up-regulation | Tbd | (CDDP)-resistant H1299 cells | Wu et al., (2020) |
|        | Cisplatin (CDDP)  | Up-regulation | Tbd | | |
|        | Potassium chloride (KCl) | Down-regulation | Might contribute to alternative splicing observed in schizophrenia (SCZ) | Neuronal stimulation of mouse primary cortical neurons and hiPSC-derived neurons. Reduced expression in SCZ patient tissue | Barry et al., (2014) |
|        | Serum starvation  | Down-regulation | Tbd | MEFs | Wang, Liang, et al. (2019) |
|        | Heroin and Cocaine| Up-regulation | Tbd | Human NAC | Albertson et al., (2006) and Michelhaugh et al., (2011) |
| Neat1  | Hypoxia           | Up-regulation | Tbd | Several tissues of mice exposed to hypoxia (including brain). In vitro and in vivo (mice) | Lelli et al., (2015) |
|        | Serum starvation  | Up-regulation | Tbd | MEFs | Wang, Liang, et al. (2019) |
|        | LPS               | Up-regulation | KD improves protection against LPS-induced myocardial injury. | MEFs | Wang, Liu, et al. (2019) |
3.2 | NEAT1

NEAT1 is a well-characterized lncRNA that organizes nuclear structures called 'paraspeckles'. Paraspeckles contain proteins involved in transcription and RNA processing (Clemson et al., 2009; Fox & Lamond, 2010). So far, two isoforms of NEAT1, NEAT1_1 and NEAT1_2, have been described that are spliced by alternative processing of the 3'-untranslated region (Fox et al., 2018; Naganuma et al., 2012; Yamazaki et al., 2018). NEAT1 is classified as one of the architectural RNAs (arcRNA), that function by providing essential scaffolds for the formation of nuclear bodies, with NEAT1_2 playing a predominant role in paraspeckle formation (Chujo et al., 2016; Naganuma et al., 2012). Paraspeckle formation is a tightly regulated process that occurs parallel to RNApolII transcription of NEAT1_2 and binding of several paraspeckle proteins (PSPs) to NEAT1 (Mao et al., 2011; Yamazaki et al., 2018). So far, more than 60 PSPs have been identified, most of which are RBPs (Yamazaki & Hirose, 2015). RNAi studies identified seven essential PSPs that are able to form a paraspeckle with ~50 NEAT1_2 molecules (Chujo et al., 2017; Naganuma et al., 2012). Six of the seven essential PSPs possess prion-like-domains, of which FUS and RNA-binding motif protein 14 are shown to be required to form paraspeckles in vivo (Hennig et al., 2015; Yamazaki & Hirose, 2015). Functionally, PSPs interact with specific domains of NEAT1 in the formation of paraspeckles. These domains were elucidated by systemic deletion of parts of NEAT1_2 using CRISPR/Cas9-mediated genome editing, revealing domains important for: 1) RNA stabilization of NEAT1_2 (C-terminal domain and N-terminal TH structure), 2) paraspeckle assembly (middle domain) and 3) isoform switching (polyadenylation signal containing domain; Yamazaki et al., 2018). The middle domain of NEAT1_2 further contains redundant functional subdomains responsible for paraspeckle assembly. By performing CLIP-seq it was confirmed that PSPs non-PoU domain-containing octamer-binding protein (NONO), splicing factor proline/glutamine-rich (SFPQ) and FUS bind several of the NEAT1_2 subdomains. Paraspeckle assembly could be rescued by artificial tethering of a NEAT1_2 mutant lacking functional subdomains with one of the PSPs (NONO, SFPQ or FUS). The phase-separation property of paraspeckles was shown by treatment with 1,6-hexanediol, which not only disrupted paraspeckle structure but also disturbed interactions between NONO and RBPs that contain prion-like-domains like FUS. It is speculated that FUS through its PLD recruits several PSPs, leading to the formation of FUS oligomers thereby mediating paraspeckle assembly via phase separation (Yamazaki et al., 2018). Overall, these studies highlight the key role of NEAT1_2 subdomains in paraspeckle assembly through phase separation.

Several lines of experimental evidence suggest that the size and number of paraspeckles is influenced by the expression level of NEAT1. Physiologically, NEAT1 and paraspeckles are not detectable in human embryonic stem cells, but differentiation leads to the induction of NEAT1 expression and formation of paraspeckles. Knockdown of NEAT1 in HeLa cells leads to the loss of paraspeckles and increased nucleocytoplasmic export of mRNAs bearing...
| Name            | ncRNA Type | Disease | Regulation                  | Function                                                                 | References                                                                 |
|-----------------|------------|---------|-----------------------------|--------------------------------------------------------------------------|----------------------------------------------------------------------------|
| NEAT1           | IncRNA     | ALS     | Up-regulated at early stage | Regulates paraspeckle formation, increased NEAT1 expression leads to neurotoxicity | Clemson et al., (2009); Nishimoto et al., (2013) and Suzuki et al., (2019) |
| C9ORF72-AS      | antisense RNA | ALS     | Up-regulated                | Forms RNA foci that recruit RBP s, DPR protein formation via repeat-associated non-ATG-initiated (RAN) translation leading to neurotoxicity | Cheng et al., (2019); Mizielska et al., (2014); Mori, et al. (2013); Sareen et al., (2013); Swinnen et al., (2018) and Wen et al., (2014) |
| ATXN2-AS        | antisense RNA | ALS     | Up-regulated                | Repeat expansion RNA induces neurotoxicity                               | Li, Sun, et al. (2016)                                                                 |
| SMN-AS          | antisense RNA | SMA     | Up-regulated                | Recruits polycomb repressive complex 2 (PRC2) complex to the SMN gene to suppress SMN expression | d’Ydewalle et al., (2017) and Woo et al., (2017)                                                                 |
| ZEB1-AS, ZBTB11-AS | antisense RNA | ALS     | Up-regulated in blood samples (peripheral blood mononuclear cells [PBMCs]) | Tbd                                                                        | Gagliardi, et al. (2018)                                                                 |
| UBXN7-AS, ATG10-AS, ADORA2A-AS | antisense RNA | ALS     | Up-regulated in blood samples (PBMCs) | Tbd                                                                        | Gagliardi, et al. (2018)                                                                 |
| hsa_circ_0001173, hsa_circ_0043138, hsa_circ_0088036 | circRNA | ALS     | Up-regulated in blood samples (PBMCs) | Biomarker potential                                                        | Dolinar et al., (2019)                                                                 |

Abbreviations: ATXN2, Ataxin-2; NEAT1, nuclear-enriched abundant transcript 1; SMN, survival motor neuron; Tbd, to be determined.
Alu repeats, implicating NEAT1 function in mRNA export (Chen & Carmichael, 2009). Induction of NEAT1 levels concomitant with paraspeckle enlargement has been observed under different conditions, for example, during viral infection and myotube differentiation (Sunwoo et al., 2009; Zhang et al., 2013). Further, proteasome inhibition in HeLa cells by MG132 led to strong up-regulation of NEAT1 together with the elongation of paraspeckles and sequestration of the transcription factor SFPQ by NEAT1. In contrast, NEAT1 knockout mouse embryonic fibroblasts display the loss of paraspeckles and are more susceptible to proteasome inhibitor-mediated cell death, suggesting a pro-survival role for paraspeckles under stressful conditions (Hirose et al., 2014).

As indicated above, NEAT1 interacts with several RBPs involved in ALS (i.e. FUS and TDP-43), both in human brain tissue and in cultured cells (Nishimoto et al., 2013; Tollerv et al., 2011). In early onset ALS patient tissue, not only the frequency of paraspeckle formation is highly increased but also the co-localization of NEAT1 with TDP-43 and FUS in paraspeckles is enhanced. Further, by performing interaction and localization studies it was concluded that NEAT1 acts as a scaffold for these RBPs in the nuclei of ALS MNs (Nishimoto et al., 2013). Subsequent work confirmed these observations, showing an increase in paraspeckle formation in the spinal cords of sALS and FALS patients (Shelkovnikova et al., 2018). ALS-associated RBPs not only interact with NEAT1 but also regulate NEAT1 expression. Deletion of FUS, TDP-43 or Matrin 3 (MATR3) leads to increased NEAT1_2 RNA levels (Banerjee et al., 2017; Naganuma et al., 2012). Paraspeckles are known to exert anti-apoptotic activity and can increase cell viability under stressful conditions (Hirose et al., 2014; Shelkovnikova et al., 2018). Their increase at early disease stages in ALS could hint at compensatory mechanisms to increase MN survival. Mutations in FUS lead to impaired paraspeckle formation by dysregulating NEAT1 transcription and mis-assembly of other paraspeckle proteins that are required during adequate stress responses (Shelkovnikova et al., 2014). This disrupts neuroprotective mechanisms and may contribute to the aggressive disease phenotype (early onset and fast progression) observed in FUS-ALS cases (An et al., 2019). Although these studies suggest that ALS-related RBP mutations affect NEAT1 expression and may thereby influence paraspeckle formation, the precise role of NEAT1 in ALS pathogenesis is incompletely understood. A recent study shows that the activation and up-regulation of endogenous NEAT1 levels using the CRISPR-Cas9 system in NSC-34 cells induces neurotoxicity. NEAT1_2, but not NEAT1_1, was proposed to mediate this neurotoxic effect (Suzuki et al., 2019). These data support the idea that altered NEAT1 expression in ALS leads to defects in paraspeckle formation causing cell death and neurodegeneration (Figure 1c). However, a neuroprotective role for NEAT1 is proposed in Parkinson’s disease (PD) (Simchovitz et al., 2019). Increased NEAT1 levels were found in the substantia nigra of PD patients and in drug-induced oxidative stress in vitro PD models. This increase in expression was also associated with formation of NEAT1 paraspeckles both in vitro and in vivo. Further, neuroprotective agents, like fenofibrate and simvastatin, were found to induce NEAT1 expression, whereas NEAT1 depletion increased oxidative stress-induced cell death (Simchovitz et al., 2019).

Several of the genes associated with ALS give rise to sense or antisense RNAs which are not translated into protein but act as IncRNAs. These include naturally occurring (anti)sense RNAs but also RNAs that gain IncRNA functions as a result of genetic changes (e.g. repeat expansions). A few prominent examples are discussed below.

### 3.3 | C9ORF72

As indicated in previous sections, the expansion of a repeat region of six-nucleotide motifs (GGGCCn (G,C)n) in the 5’ region of the C9ORF72 gene is the most common genetic cause of ALS and frontotemporal dementia (FTD; DeJesus-Hernandez et al., 2011; Renton et al., 2011; Zu et al., 2013). FTD is a form of dementia characterized by impaired judgement and executive skills, and loss of neurons in the frontal and temporal cortices (Burrell et al., 2016). ALS and FTD show overlapping disease phenotypes both at the symptomatic and genetic levels. The (G,C)n repeat expansions in C9ORF72 are observed in three transcript variants: in variants 1 and 3 the expansions are located in intron 1 and in variant 2 they are present in the promoter region (Balendra & Isaacs, 2018). These C9ORF72 repeat expansions are not only found in almost 40% of familial ALS and FTD cases, but also in up to 8% of sporadic cases of ALS and FTD (DeJesus-Hernandez et al., 2011; Majounie et al., 2012; Rademasifers, 2012; Renton et al., 2011). Healthy individuals carry up to 20–30 copies of the repeat, whereas repeat size increases to hundreds to thousands of (G,C)n sequences in ALS patients (DeJesus-Hernandez et al., 2011). C9ORF72 loss-of-function and toxic gain-of-function mediated by the repeat expansions have been implicated in C9ORF72-associated ALS cases (C9-ALS).

In addition to different sense isoforms of C9ORF72, one or more antisense transcripts that arise from the same promoter have been detected. The mutant hexanucleotide repeat regions of C9ORF72 are transcribed in a bidirectional fashion, producing sense (C9ORF72-S), with G,C repeats, and antisense (C9ORF72-AS), with GGGCC (G,C)n repeats, RNAs (Donnelly et al., 2013; Mizielsinska et al., 2013; Mori et al., 2013; Renton et al., 2011). Both expanded repeat-containing transcripts (sense and antisense) can be translated into poly-dipeptides (dipeptide repeat [DPR] proteins) which accumulate in MNs of C9-ALS patients (Figure 1c; Freibaum & Taylor, 2017; Gendron et al., 2017; Mori et al., 2013; Saberi et al., 2018; Wen et al., 2014; Zu et al., 2013). Several studies have focused on understanding the function of C9ORF72-S RNA and its associated proteins (reviewed in Moens et al., 2017). Over-expression of G,C repeats in cell culture, C. elegans, Drosophila melanogaster led to deleterious phenotypes, which provided evidence that C9ORF72-associated toxic gain-of-function contributed to the disease (Burguete et al., 2015; Freibaum et al., 2015; Kramer et al., 2016; Lee et al., 2013; Mizielsinska et al., 2014; Wen et al., 2014; Xu et al., 2013; Zu et al., 2011). Similarly, adeno-associated virus-mediated over-expression of 66 G,C repeats in mice led
to an accumulation of RNA foci and DPR proteins together with neuronal cell loss, TDP-43 pathology and cognitive and motor dysfunction (Chew et al., 2015). The functional relevance of C9ORF72-AS is less well understood. Interestingly, C9ORF72-AS RNAs harbour a few miRNA-binding sites in the first exon (Douglas, 2018). Similar to C9ORF72-S, C9ORF72-AS forms RNA foci in MNs and sporadically in interneurons (in frontal cortex and spinal cord) of ALS and FTD patients (DeJesus-Hernandez et al., 2011; Gendron et al., 2013; Mizielinska et al., 2013; Zu et al., 2013). However, a higher frequency of C9ORF72-AS as compared to C9ORF72-S RNA foci and DPR proteins is observed in MNs of C9-ALS patients (Cooper-Knock et al., 2015). C9ORF72-AS RNA foci are found in the peri-nucleolar regions, suggesting that nucleolar defects or stress may contribute to C9-ALS pathogenesis (Kwon et al., 2014; Tao et al., 2015). It is important to note, however, that RNA foci formed by either C9ORF72-S or C9ORF72-AS do not correlate with the extent of neurodegeneration observed in ALS patients and are not determining factors of the clinico-pathological variability observed in C9-ALS cases (DeJesus-Hernandez et al., 2017). This may imply a role for other toxic modifiers in disease pathogenesis.

C9ORF72-S expansions are reported to form G-quadruplexes, which regulate transcription and gene expression (Fratta et al., 2012; Haeusler et al., 2014; Reddy et al., 2013; Wang, Goodrich, et al., 2019). C9ORF72-AS repeats do not form G-quadruplexes but can form i-Motifs and hairpin structures, that can affect genome stability and transcription (Assi et al., 2018; Kovanda et al., 2015). Further, the crystal structure of C9ORF72-AS repeat RNA showed that C9ORF72-AS RNA forms a double helix structure with tandem C:C mismatches, that could attract proteins recognizing these structures (Dodd et al., 2016). Therefore, repeat expansions in C9ORF72 may not only affect ALS pathogenesis through the formation of RNA foci or production of DPR proteins, but also indirectly by regulating the secondary structure of DNA and RNA, and thereby gene expression.

To determine how ALS-associated C9ORF72 repeat expansions cause ALS, different in vivo models have been generated. Expression of C9ORF72 repeat expansions that form DPR proteins in Drosophila is toxic leading to adult-onset neurodegeneration with reduced lifespan (Mizielinska et al., 2014). Expression of repeat expansion RNA that is not translated into DPR proteins did not induce neurotoxicity, suggesting that the toxic effect of C9ORF72 repeat expansions may be mediated by DPR proteins (Mizielinska et al., 2014). To better understand the role of C9ORF72-S and C9ORF72-AS repeat RNA expansions, another study created RNA-only Drosophila models, to avoid the effects of DPR proteins. Flies were generated to express 1) small (~100) RNA repeats in sense or antisense directions either as a processed polyadenylated transcript (named polyA) or as intronic versions fused with green fluorescent protein exons, or 2) large RNA-only repeats (>1,000) in the sense direction (Moes et al., 2018). Both, sense and antisense polyA repeat RNA was found to accumulate in the cytoplasm of adult Drosophila neurons, whereas intronic RNAs with small and large repeats accumulated in the nucleus forming RNA foci, mimicking pathological conditions. Even though the RNA foci sequestered endogenous Drosophila RBPs, no neurodegenerative phenotypes or toxicity were observed following either sense or antisense RNA expression (small and large repeats). This suggests that mutant RNA is not sufficient to induce disease-relevant toxicity (Moens et al., 2018). However, expression of repeat expansions in a Danio rerio (zebrafish) model induced neurodegenerative phenotypes (Swinnen et al., 2018). Injection of ~70 C9ORF72-S (G₄C₄) or C9ORF72-AS (G₄C₄) repeats in zebrafish embryos induced motor axonopathy. A dose-dependent effect was observed on axon outgrowth and branching. Further, it was shown that toxicity induced by repeat RNA was independent of DPR proteins, suggesting that the presence of repeat RNA in the absence of DPR proteins is sufficient to induce MN toxicity in zebrafish (Swinnen et al., 2018). These differences in sensitivity for RNA-induced toxicity may be explained by species differences or differences in experimental approaches. However, even within one species (mouse) different effects of the expression of repeat RNAs were found. Even though in most models RNA foci and DPR protein inclusions formed from both sense and antisense C9ORF72 RNAs were observed, some models developed motor or cognitive defects, whereas others were unaffected (Jiang, Zhu, et al., 2016; Liu et al., 2016; O’Rourke et al., 2015; Peters et al., 2015). Further work is needed to carefully assess the role of RNA toxicity in C9-ALS. Expression of longer repeat sequences (in the pathological range) may be required to model RNA toxicity accurately.

Despite this recent progress, the precise effects of the RNA foci or DPR proteins observed in C9-ALS are still unclear. Although valuable, studies on post-mortem tissue from patients do not reveal early pathogenic events and experimental work provides conflicting conclusions. The inconsistencies in recapitulating toxicity observed in ALS C9ORF72 gain-of-function models could be because of the: (a) methodological difficulties in cloning and expressing GC-rich repeats at a length that is observed in patients leading to inconsistent mimicking of repeat pathology; (b) over-expression of repeat expansions in different in vitro and in vivo models, which may not necessarily mimic the endogenous levels of the expanded C9ORF72-S or -AS transcripts observed in patients; and (c) sequestration of a different set of RBPs by RNA foci in humans and different experimental models (Balendra & Isaacs, 2018).

Targeting of C9ORF72-S by antisense oligonucleotides (ASOs) and of the G-quadruplex structure formed by C9ORF72-S by specific binding molecules have been explored as therapeutic strategies for C9-ALS (Donnelly et al., 2013; Jiang, Zhu, et al., 2016; Lagier-Tourenne et al., 2013; Simone et al., 2018; Zamarri et al., 2014; Zhang et al., 2015). ASOs were effective in reducing RNA foci and reversed gene expression changes in C9-ALS hiPSC-derived MNs (Sareen et al., 2013). Even specific targeting of the repeat expansion by ASOs mitigated pathological hallmarks of ALS. In addition to reducing intranuclear accumulation of C9ORF72-S in RNA foci and normalizing dysregulated gene expression, ASOs were effective in reducing sequestration of the RBP adenosine deaminase RNA-specific B2 (inactive) to the repeat expansion and in reversing glutamate toxicity in C9-ALS patient fibroblasts and neurons (Donnelly et al., 2013).
However, ASOs targeting the sense strand were not effective in reducing the abundant RNA foci formed by C9ORF72-AS (Lagier-Tourenne et al., 2013). This could limit clinical efficacy if toxicity in patients is mainly caused by C9ORF72-AS RNA. Another important consideration for ASO therapy are off-target effects. The application of ASOs could lead to adverse side effects, because of their off-target effects depending on the sequence and modification chemistry of the motifs used to stabilize the ASO-molecule for protection against cellular degradation (Schoch & Miller, 2017). Off-target effects of ASOs are caused by binding to other mRNAs leading to an undesirable biological effect and activation of the immune system. Non-specific target binding is avoided by careful selection of candidate ASOs. Human clinical ASO candidates are routinely screened in silico for potential mismatches and tested empirically for their effect on mRNAs (Kamola et al., 2015; Monia et al., 1992). Activation of the immune system is because of the recognition of ASOs with certain chemical modifications as foreign DNA by toll-like receptors. It is reported that, unmethylated cytosine-phosphorus-guanine motifs in the ASO are recognized by toll-like receptors-9, resulting in antibody production, activation of T lymphocytes, natural killer cells and release of cytokines, interleukins (IL)-6, IL-12 and interferon (INF)-γ (Agrawal & Kandimalla, 2004; Karaki et al., 2019; Vollmer et al., 2004). However, adaptations in the ASO design have largely mitigated this effect (Bennett & Swayze, 2010; Scoles et al., 2019; Sewell et al., 2002). Despite extensive research in ASO development, unrecognized off-target issues remain (Schoch & Miller, 2017). So, it is important that each ASO molecule is evaluated independently and considered for a complete toxicological assessment.

Different effects of antisense transcripts on their sense counterparts have been described (Kanduri, 2008; Khorkova et al., 2014; Malecová & Morris, 2010; Modarresi et al., 2012; Yu et al., 2008). First, formation of double-stranded RNA structures by hybridization of overlapping complementary RNA sequences in sense and antisense transcripts can activate the RNA interference pathway (Polikarpov, 2013; Werner et al., 2014). Second, antisense transcripts may cause transcriptional interference via the displacement of sense transcription factors in the sense promoter region (Wight & Werner, 2013). Third, antisense transcripts may epigenetically regulate sense transcripts through recruitment of chromatin remodelling factors and modulation of their activity (Kanduri, 2008; Malecová & Morris, 2010; Yu et al., 2008). Given these possible roles of antisense transcripts, sense oligonucleotides (SOs) designed against antisense transcripts may be a valuable therapeutic strategy. However, further studies are needed to better define the role of C9ORF72-AS in C9-ALS.

### 3.4 ATAXIN

The ALS risk gene ataxin-2 (ATXN2) also gives rise to antisense transcripts, ATXN2-AS, with increased expression in ALS patient tissue (Li, Sun, et al., 2016). ATXN2 protein is ubiquitously expressed in neuronal and non-neuronal tissues and is localized to the cytoplasm (Huynh et al., 1999). ATXN2 associates with polyribosomes in the endoplasmic reticulum and regulates mRNA translation (Satterfield & Pallanck, 2006). Further, it influences mRNA maturation, energy metabolism and endocytosis (Huynh et al., 1999; Magaña et al., 2013). In ALS, mutant ATXN2 interacts with FUS and TDP-43 in an RNA-dependent interaction manner which contributes to disease pathogenesis (Elden et al., 2010; Van den Heuvel et al., 2014; Yokoshi et al., 2014). The interaction of ATXN2 and TDP-43 was first identified in a screen for toxic modifiers of TDP-43. This work showed that an increase in the length of a polyQ repeat in ATXN2 (from 22 normal to 27–33 glutamines in ALS) is significantly associated with enhanced risk for developing ALS (Elden et al., 2010). ATXN2 polyQ repeat expansions of more than 33 repeats are associated with another neurodegenerative disease spinocerebellar ataxia type 2 (SCA2; Li, Sun, et al., 2016; Van den Heuvel et al., 2014). SCA2 is an autosomal-dominant disorder caused by the expansion of CAG repeats in the N-terminal coding region of ATXN2. Clinical features of SCA2 are characterized mainly by limb and gait ataxia, dystar-thria and abnormal eye movements (Cancel et al., 1997; Geschwind et al., 1997). The symptoms of SCA2 are found to be clearly of cerebellar origin and involve cerebellar circuits. However, a typical ocular-dependent feature is also observed in SCA2, where slow or absent saccades are displayed arising from the degeneration of oculomotor neurons. Other frequently observed symptoms include dystonia, myoclonus as well as neuropathy, muscle spasticity and frontal-executive dysfunction (Geschwind et al., 1997; Scoles & Pulst, 2018).

The ATXN2 locus is bidirectionally transcribed in both ALS and SCA2 patients carrying an ATXN2 repeat expansion. In human ALS lymphoblastoid lines a non-translatable ATXN2-AS transcript was detected from both the normal and expanded allele (with intermediate CAG expansions [31–32 triplets]). Toxicity assays were performed to confirm the toxic nature of the expanded ATXN2-AS transcript in SK-N-MC neuroblastoma cells (Li, Sun, et al., 2016). These mutant CAG/CUG transcripts aberrantly interact with RBPs involved in, for example, ribosomal RNA (rRNA) processing and splicing (Figure 1c; Swinnen et al., 2020). Despite these promising results, whether and if so how ATXN2-AS contributes to ALS pathogenesis remains to be established.

### 3.5 Other IncRNAs in ALS

Whole transcriptome RNA sequencing (RNA-seq) analysis in peripheral blood mononuclear cells (PBMCs) and spinal cord from sALS and fALS patients identified several differentially expressed IncRNAs (Gagliardi, et al., 2018). Several of these IncRNAs were sense RNAs that were deregulated in sALS, for example, ZEB1-AS and ZBTB11-AS, and with sense counterparts that have reported functions in transcription regulation (Keightley et al., 2017; Li, Xie, et al., 2016). Interestingly, a few of these antisense IncRNAs can potentially target genes associated with neurodegenerative diseases, that is, UBXN7-AS, ATG10-AS and ADORA2A-AS (Guo & Qi, 2017; Lee et al., 2015; Villar-Menéndez et al., 2014). The detection of IncRNAs...
in PBMCs and blood raises the possibility that lncRNAs may be used as biomarkers for assessing clinical disease course or characteristics. Intriguingly, several of the lncRNAs detected in healthy and diseased MNs have also been reported in blood, PBMCs or peripheral blood lymphocytes, for example, NEAT1, MALAT1, MEG3 and ZEB1-AS1 (Fallah et al., 2019; Gagliardi et al., 2018; Gao et al., 2016; Huang et al., 2017; Wang, Li, et al., 2019).

Other lncRNAs have been implicated in ALS (or FTD) through their link with proteins associated with ALS pathogenesis, for example, TDP-43 or FUS. MALAT1 showed increased expression and binding to TDP-43 in cortical tissue of sporadic FTD patients, while MEG3 was down-regulated and showed decreased TDP-43 binding (Tollervey et al., 2011; Zhang et al., 2017). TDP-43 can associate with several other lncRNAs, including BDNFOS and TFE6α (in SHSY5Y cells) and Myolinc (in muscle cells; Militello et al., 2018; Xiao et al., 2011). Expression of the lncRNA heat-shock ma ω (hsω) is positively regulated by TDP-43, with direct binding of TDP-43 to the hsω locus (Chung et al., 2018). The stress-induced lncRNA Satellite III repeat RNA (Sat III) is the human orthologue of Drosophila hsω and shows increased expression in human FTD patient tissue and in cellular models of TDP-43 over-expression (Chung et al., 2018).

The introduction of ALS-associated mutations in FUS in mESC-derived MNs affects the expression of several lncRNAs. A specific up-regulation of the lncRNA Lhx1os and down-regulation of LncMN-1 (2610316D01Rik) and LncMN-2 (5330434G04Rik) were observed in FUS±/±/L17 and FUS±/− MNs, indicating that FUS loss-of-function affects lncRNA expression. Lhx1os, LncMN-1 and LncMN-2 are conserved between mouse and human, displaying increased expression during human in vitro MN differentiation (Biscarini et al., 2018). The Drosophila orthologue of human FUS, dFUS, interacts with hsω. Depletion of hsω leads to cytoplasmic mis-localization and loss of nuclear dFUS function. Further, MN-specific knockdown of hsω impairs locomotion in larval and adult flies, and also induces anatomical defects in pre-synaptic terminals of MNs (Lo Piccolo & Yamaguchi, 2017). Given these diverse interactions of TDP-43 and FUS with lncRNAs it is likely that the cytoplasmic mis-localization and perturbed functions of TDP-43 and FUS in ALS MNs impacts on lncRNA distribution, expression and/or function that may contribute to MN degeneration and ALS.

### 3.6 Spinal muscular atrophy (SMA)

SMA is a neurodegenerative disease characterized by profound muscle weakness attributed to progressive spinal MN degeneration, observed within weeks to months after birth or even at birth. SMA is classified as a rare disease that has a worldwide incidence of one in 6,000 – 10,000 newborns. However, it is the second most common autosomal recessive disease after cystic fibrosis and is the most common monogenic defect leading to infant mortality (Lunn & Wang, 2016). SMA is caused by reduced levels of SMN protein because of recessive mutations of the SMN1 gene and retention of a variable copy number of the highly homologous SMN2 gene (Lefebvre et al., 1995; Lorson et al., 1999). The critical sequence difference between SMN2 and SMN1 is one nucleotide (C → T) in exon 7 at a splice enhancer site. This change results in alternative splicing of SMN2 pre-mRNA (Lorson et al., 1999; Monani et al., 1999). This leads to a preferential exclusion of exon 7 from most transcripts (SMN1Δ7), leading to a truncated unstable SMN protein, contributing to reduced levels of overall functional SMN protein. Individuals affected by SMA show loss of SMN protein and carry at least one copy of SMN2. The number of SMN2 copies inversely correlates with disease severity (Lefebvre et al., 1995). In addition to SMN2 copy number, other modifiers are known to modulate SMA clinical severity (Crawford et al., 2012). Therapy development for SMA has focused on increasing SMN expression and, as outlined below, lncRNAs may be valuable targets for modulating SMN expression. A lncRNA that arises from the antisense strand of SMN, SMN-AS1, represses SMN expression (d’Ydewalle et al., 2017; Woo et al., 2017). Similar to what is described for C9ORF72-AS and ATXN2-AS, SMN-AS1 is a naturally occurring antisense transcript. SMN1-AS mainly localizes to neural tissues and neurons, and its expression increases during neuronal differentiation, inversely correlating with SMN protein expression. Chromatin immunoprecipitation showed that SMN1-AS mediates the recruitment of PRC2 to the SMN2 promoter, suppressing SMN protein expression. This effect can be rescued by knockdown of SMN1-AS or inhibition of PRC2 activity (d’Ydewalle et al., 2017; Woo et al., 2017). Targeting SMN1-AS using ASOs together with SMN2 splice-switching oligonucleotides increases SMN expression in a dose-dependent manner and improves survival in a severe mouse model of SMA (d’Ydewalle et al., 2017). These studies provide an important proof-of-concept for targeting lncRNAs as a means of inducing SMN expression and modulating disease progression.

### 3.7 CircRNAs in motor neuron disease

Several studies have investigated circRNAs in the context of human disease (Chen, Li, et al., 2016). In recent years, transcriptome analyses have revealed that dysregulation of circRNAs is associated with complex neurological diseases such as Alzheimer’s disease, PD, multiple system atrophy and ALS (Chen, Mills, et al., 2016; D’Ambra et al., 2019; Errichelli et al., 2017; Hanan et al., 2020; Huang et al., 2018; Wang et al., 2018). As outlined in previous sections, FUS is required for circRNA biogenesis (Errichelli et al., 2017). HiPSC-derived MNs harbouring ALS-linked FUS mutations (FUS-P525L) also showed deregulation of specific circRNAs suggesting that these mutations affect circRNA biogenesis. CircRNAs may not only play a role in ALS pathogenesis but could also be exploited as therapeutic agents. A genome wide loss-of-function screen for modifiers of TDP-43 toxicity identified (intronic) lariats as decoys for TDP-43. Reduction of debranching enzyme 1 activity led to an increase in intronic lariats in this screen. The newly formed lariats acted as decoys and sequestered toxic cytoplasmic TDP-43 aggregates, possibly preventing them from interacting with other RNAs and RBPs (Armakola et al., 2012). This study suggests the possibility of using
circular lncRNAs (such as circRNAs) as decoys for toxic proteins in ALS. circRNAs were also investigated as potential biomarkers in ALS using microarray analysis (Dolinar et al., 2019). Expression profiling of circRNAs in ALS was performed by comparing leukocyte samples from 12 patient and eight age- and sex-matched healthy controls. Interestingly, several of the dysregulated circRNAs were located in gene loci that play key roles in ALS pathologies, for example, hsa_circ_0001173 in vesicle-associated membrane protein-associated protein B/C (Nishimura et al., 2004). Three of the circRNA candidates (hsa_circ_0023919, hsa_circ_0063411 and hsa_circ_0088036) showed similar differential expression patterns in a replication experiment as in the original microarray experiment. Where, hsa_circ_0023919 was down-regulated, hsa_circ_0063411 and hsa_circ_0088036 were up-regulated in ALS patients, with good sensitivity and specificity for detection. This suggests that these candidate circRNAs could be used as diagnostic markers for sALS (Dolinar et al., 2019). While microarrays only allow detection of selected RNAs, RNAseq approaches can facilitate a more unbiased detection of circRNAs in the future, including of low abundant and novel transcripts, and in identification of their biomarker potential.

Overall, these studies hint at roles for circRNAs in the pathogenesis of ALS, and as diagnostic and therapeutic targets.

4 | CONCLUSION

During the past years, a large body of experimental evidence has highlighted the role of lncRNAs in MN development. Upregulation of the expression of linear and circular lncRNAs during MN development, conservation of these RNA molecules across species and their involvement in polycomb repressive complex 1/2 recruitment and function are common observations. Interestingly, often the same cluster of lncRNAs that is regulated during MN development is dysregulated in a MND context (e.g. Lhx1os, IncMN-1, IncMN-2). These results suggest that lncRNAs are essential for normal MN development and that their dysregulation could underlie the pathogenesis of diseases such as ALS and SMA. The main challenge in the field is to identify ‘key’ downstream targets that are affected by lncRNA dysregulation and that lead to MN degeneration. Future work should focus on downstream targets that go beyond the direct interactors of lncRNAs but that instead unveil underlying molecular networks. For this, development of both genetic tools and innovative in vitro/in vivo models is essential. Taking advantage of iPSC technology and genome editing for generating patient-derived models (and isogenic controls) will be the key for dissecting the different roles of lncRNAs. Knockdown approaches, co-immunoprecipitation and argonaute 2-CLIP experiments combined with proteomics and next-generation sequencing analysis of lncRNAs are part of the toolbox that could reveal the nature and consequences of lncRNA interactions. Such experimental strategies would allow the establishment of a direct link between lncRNA-related targets and functions and consequent phenotypic alterations in MNs. Such improved understanding of the mechanism-of-action and roles of lncRNAs will open up exciting ideas for molecular strategies for treating MNDs. An interesting hypothesis that deserves further investigation is the idea that lncRNAs may contribute to the selective vulnerability of specific subtypes of MNs. Other classes of ncRNAs, including miRNAs, can mediate the subtype-specific resistance of MNs to degenerate (Hoye et al., 2017; Tung et al., 2019). For example, reduced expression of the miR-17–92 cluster of miRNAs in limb innervating lateral motor column-motor neurons (LMC-MNs) is associated with increased vulnerability of LMC-MNs to degenerate (Kanning et al., 2010; Tung et al., 2019). LncRNAs may subserve similar functions, especially given their ability to influence miRNA function. Sex-specific expression of IncRNAs also has been reported and it is interesting from a disease perspective as MNDs such as ALS and X-linked SMA occur more frequently in males as compared to females (Dressman et al., 2007; Wijesekera & Leigh, 2009). Currently, not much is known about potential sex-specific expression of the IncRNAs but establishing such expression patterns would be the first step to probe sex-specific lncRNA functions in healthy and diseased MNs.

Overall, accumulating experimental evidence hints at important roles for IncRNAs in MN development and disease. Although in many instances further work is needed to dissect how IncRNAs function in developing MNs and why lncRNA dysregulation contributes to MNDs, it is clear that such knowledge could aid the development of diagnostic and therapeutic approaches for treating MN pathologies like ALS and SMA.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

ORCID

Vamshidhar R. Vangoor https://orcid.org/0000-0003-0751-9772
Andrea Gomes-Duarte https://orcid.org/0000-0001-6079-1906
R. Jeroen Pasterkamp https://orcid.org/0000-0003-1631-6440

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