

## REVIEW ARTICLE

# Opportunities for histone deacetylase inhibition in amyotrophic lateral sclerosis

Yvonne E. Kling<sup>1,2</sup>  | Donya Pakravan<sup>1,2</sup>  | Ludo Van Den Bosch<sup>1,2</sup> 

<sup>1</sup>Department of Neurosciences, Experimental Neurology and Leuven Brain Institute (LBI), KU Leuven-University of Leuven, Leuven, Belgium

<sup>2</sup>Laboratory of Neurobiology, VIB, Center for Brain & Disease Research, Leuven, Belgium

## Correspondence

Ludo Van Den Bosch, Department of Neurosciences, Experimental Neurology, and Leuven Brain Institute (LBI), KU Leuven-University of Leuven, Leuven, Belgium.  
Email: ludo.vandenbosch@kuleuven.vib.be

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease. ALS patients suffer from a progressive loss of motor neurons, leading to respiratory failure within 3 to 5 years after diagnosis. Available therapies only slow down the disease progression moderately or extend the lifespan by a few months. Epigenetic hallmarks have been linked to the disease, creating an avenue for potential therapeutic approaches. Interference with one class of epigenetic enzymes, histone deacetylases, has been shown to affect neurodegeneration in many preclinical models. Consequently, it is crucial to improve our understanding about histone deacetylases and their inhibitors in (pre)clinical models of ALS. We conclude that selective inhibitors with high tolerability and safety and sufficient blood-brain barrier permeability will be needed to interfere with both epigenetic and non-epigenetic targets of these enzymes.

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## KEYWORDS

amyotrophic lateral sclerosis, HDAC, HDAC inhibitor, histone deacetylase, motor neuron, neurodegeneration

**Abbreviations:** ALS, amyotrophic lateral sclerosis; BBB, blood-brain barrier; C9orf72, chromosome 9 open reading frame 72; CBP, CREB-binding protein; FUS, fused in sarcoma; G3BP1, Ras GTPase-activating protein-binding protein 1; GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; HSF1, heat shock factor 1; HSP, heat shock protein; iPSC, induced pluripotent stem cell; MEF2, myocyte enhancer factor 2; MIR206, microRNA-206; miro1, mitochondrial Rho GTPase 1; NaPB, sodium phenylbutyrate; NuRD, nucleosome remodelling and deacetylase; SG, stress granule; SIRT, sirtuin; TDP-43, TAR DNA-binding protein 43; TSA, Trichostatin A; UCP1, uncoupling protein 1; VPA, valproic acid;  $\alpha$ TAT1,  $\alpha$ -tubulin acetyltransferase.

[Correction added on 10 September 2020, after first online publication: Table 1 has been corrected in this current version.]

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## 1 | INTRODUCTION

## 1.1 | Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by the progressive degeneration of upper and lower motor neurons. Upper motor neurons are located in the motor cortex, while lower motor neurons are present in the brainstem and spinal cord. The degeneration of motor neurons results in the loss of synaptic connectivity with, and signalling to, muscles causing muscle weakness and atrophy. ALS progressively leads to paralysis and eventually death, usually because of respiratory failure within

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3–5 years after symptom onset (Brown & Al-Chalabi, 2017; Van Damme, Robberecht, & Van Den Bosch, 2017). Typically, the onset is spinal, and early symptoms include weakness in the limbs. However, in approximately one third of ALS patients, the onset is bulbar and symptoms start as difficulties in swallowing or speaking (Brown & Al-Chalabi, 2017). The average age of onset is between 51 and 66 years old, but cases with an early disease onset have been described. Another impediment in the mechanistic analysis of the disease is that 90% of the cases are sporadic (sALS) compared to only 10% of familial cases (fALS) (Renton, Chiò, & Traynor, 2014). Although considerable research has been devoted to unravelling the mechanisms of ALS pathogenesis, we still do not fully understand the mechanisms that underlie ALS. One important challenge is to resolve the genetic heterogeneity in order to improve our understanding. So far, many genes involved in both familial and sporadic ALS have been identified. The major fALS-associated mutations are found in *Cu/Zn SOD1*, *TAR DNA-binding protein (TARDBP)*, *fused in sarcoma (FUS)*, and the hexanucleotide repeat expansion in *C9orf72*. Many other genes have been identified, such as *TANK-binding kinase 1 (TBK1)*, *valosin-containing protein (VCP)*, *tubulin 4A (TUBA4A)*, *ataxin 2 (ATXN2)*, and *dynactin 1 (DCTN1)*.

*SOD1* was the first to be identified as a gene associated with ALS and was discovered 27 years ago (Rosen et al., 1993). To date, over 150 mutations in *SOD1* have been described, accounting for approximately 12% of familial and 1% of sporadic cases (Andersen, 2006). The protein *SOD1* (superoxide dismutase 1) primarily protects the cell from free radicals and resides in both the cytoplasm and the mitochondrial intermembrane space. Importantly, a toxic gain-of-function and not a loss-of-function of *SOD1* has been associated with ALS (Cleveland & Rothstein, 2001). Consequently, a mutant *SOD1* mouse model has provided a platform to study potential therapeutic strategies. However, so far, all clinical studies failed to reproduce these therapeutic effects in a mostly sporadic ALS population.

*TARDBP* encodes the transcriptional repressor protein TAR DNA-binding protein 43 (TDP-43), which is involved in mRNA processing and regulation. This protein is the major constituent of the ubiquitin-positive neuronal inclusions found in both ALS (around 95% of inclusions stain positive for TDP-43) and frontotemporal dementia (FTD), a disease belonging to the same disease spectrum as ALS. *TARDBP* mutations represent about 0.9% of all ALS and about 4% of fALS cases and mainly occur in the C-terminal glycine-rich region (Al-Chalabi, Van Den Berg, & Veldink, 2017; Renton et al., 2014; Van Damme et al., 2017).

*FUS* is a DNA/RNA-binding protein that is also called translocated in sarcoma (TLS) and regulates gene expression. Mutations in *FUS* occur in 4% of fALS, resulting in neuronal and glial cytoplasmic mislocalization (Renton et al., 2014; Vance et al., 2009), and mainly occur in the C-terminal domain of the protein, containing the nuclear localization signal (Mitchell et al., 2013). Not only mutations in *FUS* but also wild-type overexpression of *FUS* has detrimental consequences on motor neurons (Mitchell et al., 2013). Despite the fact that both TDP-43 and *FUS* shuttle between the cytosol and nucleus and mislocalize to the cytoplasm,

*FUS*-immunoreactive cytoplasmic inclusions do not stain for TDP-43 or ubiquitin, highlighting the different neuropathologies occurring in ALS (Vance et al., 2009). Of particular interest is a recent study in motor neurons derived from ALS patients in which an increased synaptic accumulation of mutated *FUS* is described, suggesting a role of synaptic *FUS* in both dendritic and axonal cellular compartments. In particular, a toxic gain-of-function due to the synaptic aggregation of mutant *FUS* was proposed (Deshpande et al., 2019).

The most common ALS-causing gene is *C9orf72* which encodes chromosome 9 open reading frame 72. It represents 10–15% of all and approximately 40% of familial ALS cases (Renton et al., 2014). Although the exact function of the *C9orf72* protein is not fully understood, evidence suggests a role in the regulation of macroautophagy and membrane trafficking. The *C9orf72* gene contains an expanded GGGGCC hexanucleotide repeat in a non-coding region of the gene. This can potentially cause the disease by at least three different, non-mutually exclusive, loss-of-function and/or gain-of-function mechanisms. First, disease could be caused by reduced *C9orf72* protein expression. Second, RNA foci containing the repeats could cause cell toxicity by binding an excessive amount of RNA-binding proteins. Third, repeat-associated non-ATG (RAN) translation of sense and anti-sense repeat-containing RNA could result in aggregate formation of dipeptide repeat (DPR) proteins of which some clearly show toxicity (Mori et al., 2013; Van Damme et al., 2017).

Ample evidence suggests that ALS starts with a loss of neuromuscular junction integrity, as shown in both patients and animal models (Fischer et al., 2004). Furthermore, skeletal muscles undergo futile denervation and re-innervation cycles (Loeffler, Picchiarelli, Dupuis, & Gonzalez De Aguilar, 2016), emphasizing the “dying-back” hypothesis, which proposes that the neuromuscular functions are impaired before motor neuron death occurs (Dadon-Nachum, Melamed, & Offen, 2011; Fischer et al., 2004). Due to this hypothesis, more focus has emerged towards the therapeutic potential of strategies targeting the neuromuscular junction and its protection and therefore promoting muscle re-innervation at early stages of ALS. However, it is still inconclusive whether ALS arises within the cell body or the axon of the motor neuron.

The two currently FDA-approved ALS treatments are **riluzole** (Rilutek) and edaravone (Radicava). Riluzole extends patient survival only by 2 to 3 months without improving muscle strength or function (Lacomblez, Bensimon, Meininger, Leigh, & Guillet, 1996). In 2017, the FDA approved also edaravone, after it proved to slow disease progression in a cohort of early-stage ALS patients in Japan. Riluzole is believed to work via modulation of glutamatergic neurotransmission and channel current modulations, such as **voltage-gated sodium channels**, while edaravone acts as an antioxidant. However, the full mechanism of action of both drugs is not completely clear.

ALS is a very heterogeneous disease with multiple mechanisms at play, making it very difficult to decide which mechanisms are causative and which result from the disease. So far, the possible pathobiological mechanisms that have been studied include glutamate excitotoxicity, oxidative stress, neuro-inflammation, mitochondrial dysfunction, axonal transport defects and altered nucleocytoplasmic

transport (Brown & Al-Chalabi, 2017; Van Damme et al., 2017). Furthermore, non-cell autonomous motor neuron death due to toxic effects by astrocytes, oligodendrocytes and microglia could play a role in ALS. Transcriptional dysregulation and epigenetics, meaning that heritable gene expression changes are induced by modifications of the DNA or the histones, has recently been emerging as a characteristic feature in the progression of ALS (Bennett, Tanaz, Cobos, & Torrente, 2019; Figueroa-Romero et al., 2012). Important players in this mechanistic pathway are **histone deacetylases** (HDACs) which might promote neurodegeneration, while there are opposing indications that HDACs could play a neuroprotective role (Morrison et al., 2006; Thomas & D'Mello, 2018). However, the mechanistic differences between the HDAC subtypes and their role in promoting or preventing neurodegeneration are still unclear. In this review, we will discuss the role of HDACs in the cell, their therapeutic potential, and their potential link with ALS in more detail.

## 1.2 | Histone deacetylases

HDACs represent a conserved class of epigenetic enzymes involved in neurodegeneration which have become a potential therapeutic target (Janssen et al., 2010; Thomas & D'Mello, 2018). In general, the highly conserved HDACs are involved in fine-tuning the transcriptional changes of the chromatin components by catalysing the deacetylation of histones, opposing their counteractors, **histone acetyltransferases** (HATs). Transcriptional inhibition is achieved by deacetylation of the histones on their lysine residues, which increases the positive charge of the histones. This ensures the interaction with the negatively charged DNA and thus "closing" the chromatin configuration. Additionally, HDACs deacetylate other proteins that are involved in cell death, the stress response, and protein degradation (Kovacs et al., 2003; Kuta et al., 2020).

There are 18 known members of the HDAC superfamily, which are further divided into four subfamilies based on sequence and structural homology. Class I, class II, and class IV contain the zinc-dependent HDACs, while class III includes the NAD<sup>+</sup>-dependent HDACs, which consists of seven sirtuins (SIRTs). Class I HDACs (HDACs 1, 2, 3, and 8) mainly reside in the nucleus. Class II HDACs shuttle between the nucleus and the cytoplasm and are further divided into class IIa (HDACs 4, 5, 7, and 9) and class IIb (HDACs 6 and 10). Class IV has only one member, HDAC11, which seems to differ in its physiological properties from the other HDACs.

## 2 | HDACs AND ALS

Rouaux et al. (2007) described hypoacetylation in a SOD1<sup>G86R</sup> model and suggested the involvement of HDACs in ALS. Since then, considerable research unravelled the major differences in expression patterns and the therapeutic potential of HDACs in ALS. Janssen et al. (2010) found **HDAC2** and **HDAC11** mRNA expression levels to be altered in post-mortem brain and spinal cord tissue of ALS patients.

However, a recent study by Dios et al. (2019) could not find HDAC differences in post-mortem patient tissue and in an in vivo PET imaging study. While the neurotoxic and neuroprotective roles of HDACs in neurodegenerative diseases have recently been reviewed (Thomas & D'Mello, 2018), our review highlights major findings of different HDAC subtypes, their inhibition, and their importance in (pre)clinical models of ALS.

### 2.1 | HDACs 1, 2, and 3

**HDAC1** mostly resides in the nucleus where it is involved in transcriptional repression (Jia et al., 2012). It is expressed primarily in neurons but is also found in glia, including astrocytes and oligodendrocytes (Broide et al., 2007). *Hdac1* deletion results in embryonic lethality in mice (Lagger et al., 2002). HDAC1 has both neuroprotective and neurotoxic effects, probably resulting from the cellular localization, the deacetylation of different targets (histone vs. non-histone substrates), and the involved interaction partners. Kim, Shanware, Bowler, and Tibbetts (2010) showed that HDAC1 can be neurotoxic upon export from the nucleus to the cytoplasm. This caused impairment of axonal transport because of HDAC1 binding to motor proteins (kinesin heavy chain members 2A and 5) and to **α-tubulin**, disrupting the complex formation with cargo proteins. Additionally, cytosolic HDAC1 was found in brain samples of patients with multiple sclerosis (Kim et al., 2010) and in an ALS FUS<sup>ΔNLS/ΔNLS</sup> knock-in mouse model (Scekic-Zahirovic et al., 2016). Recently, it was discovered that TDP-43 interacts with HDAC1 and that a reduction of HDAC1 as well as HDAC1 inhibition reduced TDP-43-mediated cell death (Sanna et al., 2020). The authors showed in both cell culture and a *Drosophila* model that a reduction as well as pharmacological inhibition of HDAC1 exerts a protective role against this TDP-43 toxicity (Sanna et al., 2020). Of particular interest is that following ER stress, nuclear transport of DNA repair-processing proteins such as p53 and HDAC1 was impaired in SOD1<sup>G93A</sup>-expressing neurons, while it localized to the nucleus in neurons without SOD1<sup>G93A</sup> (Li, Song, Moh, Kim, & Kim, 2019). Another study by Zhu and colleagues showed that nuclear export of HDAC1 was dependent on post-translational modification, that is, dephosphorylation of HDAC1, which involved calcineurin and caused the export into the cytoplasm. Genetic ablation as well as nuclear localization of HDAC1 was neuroprotective (Zhu et al., 2017). HDAC1 also interacts with **Sirt1**, an NAD<sup>+</sup>-dependent HDAC, which was neuroprotective by preserving genomic stability (Dobbin et al., 2013). Down-regulation of HDAC1 leads to DNA damage in both cultured neurons and in vivo models of neurodegeneration. Taken together, these findings outline the importance of maintaining the correct HDAC1 activity in the adult neuron (Kim et al., 2008).

Although HDAC1 and **HDAC2** show a high homology and functional overlap, they seem to be involved in different mechanisms, depending on localization and recruited interaction partners. They are both associated with the Sin3a, CoREST, and nucleosome remodelling and deacetylase (NuRD) protein complexes to regulate transcription,

DNA repair, and replication. HDAC1 and HDAC2 are essential for Schwann cell survival and myelination, inducing transcriptional factors required for myelin genes (Jacob et al., 2011).

HDAC2 is expressed at higher levels than HDAC1 and is mostly considered as being neurotoxic, fulfilling the more traditional role of histone deacetylation, including gene regulation as well as neuronal differentiation and axonal regeneration (Broide et al., 2007). *Hdac2* deletion results in postnatal lethality with cardiac defects (Montgomery et al., 2007). HDAC2 is strongly associated with learning and memory, as it reduces histone acetylation levels of and associates with genes involved in learning and memory. HDAC2 mRNA levels were found to be the highest in motor cortex and spinal cord, comparing ALS patient samples to controls (Janssen et al., 2010). Riva and colleagues compared gene expression in tissue biopsies of motor nerves from early-stage ALS patients with biopsies from motor neuropathy patients. They showed a significant down-regulation of genes related to **glutamate** metabolism and a network analysis correlated this to HDAC2. Furthermore, the authors described a significant overrepresentation of pathways related to HDAC activity, methyltransferase activity, and the NuRD complex (Riva et al., 2016).

Although **HDAC3** shares a high structural similarity with HDAC1 and HDAC2, it is the highest expressed class I HDAC in the rat brain, promoting transcriptional repression, and it is mostly implicated in neurodegeneration (Bardai & D'Mello, 2011; Broide et al., 2007). It resides in both the nucleus and the cytoplasm and is primarily expressed in neurons, but also in glia, including astrocytes and oligodendrocytes (Broide et al., 2007). Deletion of *Hdac3* is lethal in mice, indicating its importance during embryonic development (Montgomery et al., 2008). HDAC3 not only plays a role in histone deacetylation but also is involved in nuclear translocation, post-translational modifications, and interactions with disease-relevant proteins. It represents a major potential target for pharmacological inhibition in order to obtain neuroprotective effects (Thomas & D'Mello, 2018). Bardai and D'Mello reported neurotoxic effects by overexpressing HDAC3, which induced cell death in cortical and cerebellar granule neurons but not in primary kidney fibroblast cells. Furthermore, shRNA HDAC3 knockdown protected against oxidative stress and potassium deprivation-induced cell death (Bardai & D'Mello, 2011). HDAC3-induced toxicity required phosphorylation by **glycogen synthase kinase 3 $\beta$**  (GSK-3 $\beta$ ). Interestingly, knockdown of *Hdac1* reduced the neurotoxic effects of HDAC3, indicating the requirement of HDAC1 for HDAC3 to exert its neurotoxicity (Bardai & D'Mello, 2011).

## 2.2 | HDACs 4, 5, and 6

**HDAC4** is widely expressed in the brain and concentrates in the dendritic spines, but it also shuttles between the nucleus and cytoplasm (Broide et al., 2007). This process is at least partly regulated by **calcium/calmodulin-dependent kinase**-mediated phosphorylation (McKinsey, Zhang, Lu, & Olson, 2000). HDAC4 is essential for development as *Hdac4* knockout mice die in early postnatal life (Vega et al., 2004). Furthermore, HDAC4 fulfils both neuroprotective and

neurotoxic effects. Nuclear translocation of HDAC4 was shown to be protective in ischaemic stroke, while others reported that this was associated with neuronal death in an ataxia mouse model (Kassis, Shehadah, Chopp, Roberts, & Zhang, 2015; Li et al., 2012).

Following motor neuron loss, a compensatory mechanism called collateral re-innervation is initiated, which fails over time leading to progressive muscle wasting (Dadon-Nachum et al., 2011). HDAC4 and its regulator microRNA-206 (MIR206) play a crucial role in this compensatory re-innervation and in disease progression (Bruneteau et al., 2013; Echaniz-Laguna, Bousiges, Loeffler, & Boutillier, 2008; Williams et al., 2009). MicroRNAs are small non-coding RNAs that negatively regulate gene expression at the post-translational level and are decreased in post-mortem spinal cord of ALS patients, and are thus suspected of delaying disease progression (Figueroa-Romero et al., 2016). MIR206 is a key regulator of the bidirectional signalling between motor neurons and skeletal muscle fibres at neuromuscular synapses. MIR206 KO mice exhibit normal neuromuscular synapses while KO in the *SOD1<sup>G93A</sup>* mouse model accelerated disease progression, indicating that the regenerative effects were at least partly mediated through HDAC4 and FGF signalling pathways (Williams et al., 2009).

HDAC4 is overexpressed in *SOD1<sup>G93A</sup>* mice and in ALS patients (Bruneteau et al., 2013; Buonvicino et al., 2018; Pigna et al., 2019). Interestingly, HDAC4 transcripts in biopsy samples were higher in rapidly progressing compared to slowly progressing ALS and were additionally correlated with impaired muscle reinnervation. Therefore, a negative role for HDAC4 up-regulation in muscle was suggested (Bruneteau et al., 2013). However, MIR206 was up-regulated independent of progression and did not correlate with disease progression or re-innervation (Bruneteau et al., 2013). In contrast to this, Pigna and colleagues reported a neuroprotective role for HDAC4 and its expression in skeletal muscle correlated with the severity of ALS. *SOD1<sup>G93A</sup>* mice displayed significantly up-regulated HDAC4 mRNA and protein levels presymptomatically, which decreased at a symptomatic stage. Skeletal muscle-specific knockout of *Hdac4* worsened the pathological features of ALS in the *SOD1<sup>G93A</sup>* mouse model, exhibiting muscle denervation and atrophy (Pigna et al., 2019). No differences were found between the muscle-specific deleted HDAC4 mKO *SOD1<sup>G93A</sup>* mice and the *SOD1<sup>G93A</sup>* mice regarding motor neuron survival, indicating that HDAC4 deletion in muscle does not affect motor neurons in the CNS of ALS mice. Transcriptome analysis showed MIR206 down-regulation in HDAC4 mKO *SOD1<sup>G93A</sup>* mice compared to *SOD1<sup>G93A</sup>* mice. Further analysis revealed a mitochondrial uncoupler as key regulator, the upstream modulator **uncoupling protein 1 (UCP1)**, suggesting its implication in the worsening of ALS symptoms (Pigna et al., 2019). UCP1, when overexpressed in skeletal muscle, increased NMJ instability and muscle denervation (Dupuis et al., 2009).

Both HDAC4 and **HDAC5** have very little enzymatic activity and seem to interact with other HDACs in order to exert deacetylase activity. Fischle et al. (2002) demonstrated that HDAC4 and HDAC5 did not possess intrinsic enzymatic activity, in contrast to the levels of activity when associated with HDAC3 (Fischle et al., 2002). The myocyte enhancer factor 2 (MEF2) family plays a crucial role as

transcription factors in muscle and neural development and maintenance. The transcriptional activity is conserved within cell types, and Arosio and colleagues compared peripheral blood mononuclear cells of sALS and SOD1 ALS patients with those of healthy controls. Both ALS types showed a significant up-regulation of MEF2C and MEF2C mRNA, without protein level alterations. In contrast, protein distribution was changed, and MEF2 downstream targets such as **BDNF**, KLF6, and RUFY3 were significantly down-regulated in patients. Interestingly, HDAC4 colocalized with MEF2D in the nuclei, while HDAC5 was localized in the cytoplasm. However, no differences were detected in HDAC4 or HDAC5 localization in ALS patients, compared with controls (Arosio et al., 2016).

HDAC5 is abundantly expressed in both brain and peripheral tissue, being responsible for transcriptional regulation (Broide et al., 2007). *Hdac5* knockout mice, although viable, displayed cardiac problems (Chang et al., 2004). HDAC5 shuttles between the nucleus and the cytoplasm and is associated with neuroprotection (McKinsey et al., 2000). Upon nuclear export, HDAC5 stimulates axonal regrowth after injury and interacts with filamin A in the axon (Cho, Park, & Cavalli, 2015; Cho, Sloutsky, Naegle, & Cavalli, 2013). Additionally, phosphorylation of HDAC5 by **PKD1** improved axon regeneration after sensory axon injury (Cho et al., 2013). In spinal cord of both SOD1<sup>G93A</sup> and SOD1<sup>G86R</sup> mouse models, HDAC5 mRNA and protein levels decreased during disease progression (Valle et al., 2014). Additionally, HDAC5 protein levels decreased in SH-SY5Y neuroblastoma cells upon expression of SOD1<sup>G93A</sup> (Valle et al., 2014).

**HDAC6** is mainly known for the deacetylation of  $\alpha$ -tubulin, but it is also able to deacetylate many other substrates, such as **tau**, cortactin, mitochondrial Rho GTPase 1 (miro1), **HSP90**, DDX3X, peroxiredoxins, and the transcription factor  **$\beta$ -catenin** (Cook et al., 2012; Hubbert et al., 2002; Kalinski et al., 2019; Kovacs et al., 2003; Li, Zhang, Polakiewicz, & Yao, 2008; Parmigiani et al., 2008; Saito et al., 2019; Zhang et al., 2007). Mice lacking *Hdac6* are viable and develop normally but show hyperacetylated tubulin (Zhang et al., 2008). HDAC6 has been suggested to have both neuroprotective and neurotoxic effects. HDAC6 is involved in protein clearance of potentially harmful misfolded proteins by binding mono-ubiquitinated and poly-ubiquitinated misfolded proteins (Kovacs et al., 2003; Pandey et al., 2007). In addition, it exerts a neuroprotective role through aggrephagy. HDAC6 recognizes and recruits ubiquitinated misfolded proteins to the molecular motor dynein. Dynein subsequently transports these proteins to aggresomes, which are then removed by macroautophagy or by promoting clearance of ubiquitinated mitochondria through mitophagy (Kovacs et al., 2003; Lee, Nagano, Taylor, Lim, & Yao, 2010; Pandey et al., 2007). HDAC6 could also stimulate the chaperoning activity of **HSP90** via deacetylation (Bali et al., 2005). Additionally, Boyault and colleagues showed that HDAC6 promoted degradation of misfolded proteins in a mouse embryonic fibroblast model by activating heat shock factor 1 (HSF1) subsequently inducing the synthesis of heat shock proteins (HSPs). Consequently, HDAC6 could be the master regulator of cell protective responses to the formation of cytotoxic protein aggregates (Boyault et al., 2007).

HDAC6 remains mostly in the cytoplasm, and several studies proposed that HDAC6 could be a therapeutic target in neurodegeneration. Of interest is the finding that expression of mutant SOD1 increased tubulin acetylation, indicating that HDAC6 impairment could be a common hallmark of various subtypes of ALS (Gal et al., 2013). We reported that HDAC6 mRNA was increased in the SOD1<sup>G93A</sup> mouse model and that knockdown of *Hdac6* was neuroprotective. Deletion of *Hdac6* extended lifespan, while it did not delay disease onset (Taes et al., 2013). Interestingly, HDAC6 is a critical stress granule (SG) component and interacts with the Ras GTPase-activating protein-binding protein 1 (G3BP1), an essential SG protein (Kwon, Zhang, & Matthias, 2007). SGs form upon stress and consist of cytoplasmic messenger ribonucleoprotein particles, and both FUS and TDP-43 localize to these SGs (Andersson et al., 2008; Colombrita et al., 2009). It was suggested that HDAC6 is a major player of the stress response by coordinating and mediating the motor protein-dependent transport of SGs along microtubules (Kwon et al., 2007). Cohen et al. (2015) reported that TDP-43 acetylation promoted TDP-43 accumulation, linking acetylation to TDP-43 proteinopathy. Interestingly, the authors suggested that HDAC6 was the major TDP-43 deacetylase and that impaired HDAC6 was causative for TDP-43 aggregation (Cohen et al., 2015). Saito et al. (2019) showed stress-activated **CREB-binding protein** (CBP) acetylation of DDX3X, which impaired liquid–liquid phase separation and led to the formation of small SGs (Saito et al., 2019). Specifically, HDAC6 also influenced liquid–liquid phase separation (Saito et al., 2019). Recently, it was shown that acetylation of TDP-43 using different HDAC inhibitors (HDACis) (**vorinostat** and **Trichostatin A** [TSA]) induced it to phase separate into round, intranuclear annuli in which acetylated TDP-43 was found in a liquid shell together with chaperones belonging to the **HSP70** family. Acetylation of TDP-43 was driving its phase separation into these spherical annuli that formed a liquid-inside-a-liquid-inside-a-liquid (Yu et al., 2020). As a consequence, HDAC6 could play a critical role in the reaction to stress which occurs during ageing or injury.

Considering the length of the axon of motor neurons, axonal transport is a crucial, but sensitive, process that has proved to be vulnerable in ALS. This critical mechanism sustains the communication between the nucleus and the synapse. The motor proteins kinesin and dynein are essential for the axonal transport of proteins, mitochondria, lysosomes, synaptic vesicles, and other organelles along the microtubule, which can be acetylated or deacetylated by  $\alpha$ -tubulin acetyltransferase ( $\alpha$ TAT1) or HDAC6, respectively. We recently discovered axonal transport deficits in induced pluripotent stem cell (iPSC)-derived motor neurons from FUS ALS patients (Guo et al., 2017). Genetic correction of these FUS patient lines rescued the phenotype, while wild-type FUS overexpression did not induce these deficits, pointing towards a “gain-of-function” mechanism of FUS mutations in ALS. Moreover, we were also able to restore axonal transport defects upon partial knockdown of HDAC6 by antisense oligonucleotide treatment (Guo et al., 2017).

Chen et al. (2015) reported decreased HDAC6 protein levels in the SOD1<sup>G93A</sup> mouse model and disease phenotype improved upon

HDAC6 overexpression, which was obtained via lentiviral injection into the right cerebral ventricle. Others found that HDAC6 knockdown increased mutant SOD1 aggregation in cultured cells and that HDAC6 selectively interacted with mutant SOD1 via two motifs similar to the SOD1 mutant interaction region (Gal et al., 2013). In contrast to this, TDP-43 and FUS siRNA knockdown reduced HDAC6 mRNA levels, and HDAC6 mRNA associated in a complex with TDP-43 and FUS (Kim et al., 2010). It is of interest that also chemical inhibition of TDP-43 with 4-aminoquinoline derivatives reduced HDAC6 expression, highlighting once more the role of TDP-43 in mRNA stabilization and degradation (Cassel et al., 2012). Altogether, HDAC6 remains a target with both neuroprotective and neurodegenerative properties, which could be due to the different functional regions present in the enzyme. It is remarkable that HDAC6 is the only HDAC with a duplication of the deacetylase domain (Boyault et al., 2007). The ubiquitin-binding domain of HDAC6 helps to protect against proteotoxicity, while the deacetylation function might be harmful as it can induce axonal transport deficits. Altogether, these data illustrate the multifunctionality of HDAC6 (Boyault et al., 2007; Guo et al., 2017).

## 2.3 | HDACs 7–11

In contrast to other HDACs, HDACs 7–11 are expressed at relatively low levels in the brain (Broide et al., 2007). Furthermore, there is not much evidence related to the involvement of HDACs 7–11 in ALS. Mice lacking *Hdac7* die during embryonic development from cardiovascular defects (Chang et al., 2006). Ma and D'Mello showed that **HDAC7** overexpression protected against neuronal cell death in cerebellar granule neurons upon apoptosis induced by low potassium treatment. However, this neuroprotective effect was independent of its deacetylase activity (Ma & D'Mello, 2011). **HDAC8** was recently found to regulate neuronal differentiation (Katayama et al., 2018), and global knockout led to prenatal lethality due to skull instability (Haberland, Mokalled, Montgomery, & Olson, 2009). Mice lacking *Hdac9* are viable but sensitive to cardiac stress signals (Chang et al., 2004). HDRP is a truncated form of **HDAC9** with no catalytic domain, obtained through alternative splicing. HDRP was neuroprotective in cerebellar granule neurons via its interaction with HDAC1 by repressing downstream genes, which can be ameliorated via HDAC inhibition (Morrison et al., 2006). This could indicate why HDAC1 can display both neurotoxic (in association with HDAC3) and neuroprotective effects in association with HDRP. **HDAC10** represses gene transcription. Mice lacking *Hdac10* are viable, and HDAC10 showed therapeutic potential for inflammatory disorders (Dahiya et al., 2020). In T-cells, HDAC11 down-regulation resulted in missplicing of Ataxin10, a disease protein for SCA10, which is a neurodegenerative disorder displaying cerebellar dysfunction and seizures (Joshi et al., 2013). Mice lacking *Hdac11* are viable and displayed obesity resistance and showed a downstream increase of UCP1 expression and activity in brown adipose tissue (Sun et al., 2018). In SH-SY5Y neuroblastoma cells, the HDAC11 protein level was decreased upon expression of SOD1<sup>G93A</sup> protein (Valle et al., 2014). Interestingly, HDAC11 mRNA and protein level in spinal

cord increased during disease progression in SOD1<sup>G93A</sup> and SOD1<sup>G86R</sup> ALS mice (Valle et al., 2014). In addition, Janssen et al. (2010) described reduced HDAC11 mRNA levels in ALS patient biopsy samples compared to controls.

## 2.4 | Sirtuins

SIRT5 regulate protein function by NAD<sup>+</sup>-dependent post-translational modifications, thereby representing a metabolic sensor for cells. Mammals have seven SIRT5 distributed over different compartments of the cell. They can be found in the nucleus (SIRT1, 2, 6, and 7), in the cytoplasm (SIRT1 and 2), or in the mitochondria (SIRT3, 4, and 5). Altered SIRT levels have been observed in both ALS mouse models and ALS patient tissue (Körner et al., 2013; Valle et al., 2014). Metabolic disturbances are important in ALS (Vandoorne, De Bock, & Van Den Bosch, 2018), and therefore, the SIRT family might represent a potential therapeutic target for ALS.

The SIRT that is mostly associated with ALS is **SIRT1**, which is a direct deacetylase of p53. *Sirt1* knockout mice displayed developmental defects and mostly died within 1 week after birth (Cheng et al., 2003). *Sirt2* knockout mice are viable but displayed axonal degeneration and locomotor disabilities over time (Fourcade et al., 2017). In ALS, SIRT1 mRNA and protein level in spinal cord of both SOD1<sup>G93A</sup> and SOD1<sup>G86R</sup> ALS mouse models decreased over time, while **SIRT2** mRNA increased (Valle et al., 2014). In addition, SIRT1 protein was expressed at a higher level in muscle tissue of these mice, and neuroblastoma cells showed a decrease in SIRT1 protein levels upon mutant SOD1<sup>G93A</sup> expression (Valle et al., 2014). It is interesting that SIRT1 expression changed depending on the duration of the disease and even more strikingly, depended on the tissue. It was increased in muscle but decreased in spinal cord. In line with this, another study found that SIRT1 protein levels drastically increased in SOD1<sup>G93A</sup> mice, while SIRT1 protein continuously decreased over time in spinal cord of healthy mice (Herskovits et al., 2018). In addition, SIRT1 overexpression in motor neurons slowed disease progression in the SOD1<sup>G93A</sup> mouse model. However, overexpressing SIRT1 in skeletal muscle did not affect disease progression (Herskovits et al., 2018). When SIRT1 overexpressing mice were crossed with SOD1<sup>G93A</sup> ALS mice, an amelioration of the ALS phenotype through activation of the HSF1/HSP70i chaperone system was observed (Watanabe et al., 2014).

**SIRT3** protected against mitochondrial fragmentation and cell death in the SOD1<sup>G93A</sup> model (Song, Song, Kincaid, Bossy, & Bossy-Wetzel, 2013). SIRT3, 4, and 5 are involved in mitochondrial function, energy metabolism, and oxidative stress (Gan & Mucke, 2008). To the best of our knowledge, no data related to a potential role of other SIRT5 in ALS are available. For further reading on SIRT5 and their biology and implications in ALS, we refer to the review by Tang (2017).

## 3 | HDACs AND ALS

Although considerable research has been devoted to unravelling the role of HDACs in neurodegeneration, in-depth knowledge of their interplay is still lacking. However, chemical inhibition of potential

therapeutic targets in different disease models will help to further investigate the roles of the different HDACs in ALS. Next, we will describe the current status of HDACis in the context of ALS.

HDACi design is a challenging task for medicinal chemists for many reasons. The structural similarity between HDAC subtypes demands crucial considerations for design, synthesis, and their application. First, individual HDACs play different biological roles within the body, creating a high risk for undesired, non-selective, inhibition due to structural similarities. Furthermore, selectivity is critical as HDACs can exhibit both neuroprotective and neurotoxic properties, even within one disease. Additionally, the region of the body in which the HDAC is intended to be inhibited is an important consideration. Therefore, blood–brain barrier (BBB) penetrance as well as cell permeability and intracellular localization have to be considered. Taking into account that changes of SIRT1 expression depend on the disease state and on the tissue (increased in muscle, but decreased in spinal cord), the approach of targeting HDACs in different regions of the body may be an attractive option (Valle et al., 2014). Moreover, HDAC4 expression was up-regulated in rapid-progressing ALS (Bruneteau et al., 2013), while others reported muscle-specific knockout of HDAC4 worsening the disease phenotype in a mouse model. This underlines the importance for the design of subtype-specific and -selective HDACis (Pigna et al., 2019).

HDACis are commonly classified based on the chemical moiety that is chelating the zinc ion in the catalytic pocket. They consist typically of hydroxamic acids, cyclic peptides, benzamides, short-chain fatty acids, or hydrazide derivatives. Of the above-mentioned, hydroxamic acids have proven to display the highest zinc-binding affinity so far, with fast “on-kinetics.” Zhang, Zhang, Jiang, Zhang, and Song (2018) published a detailed review on HDACi design and zinc-binding groups for HDACis. Besides the zinc-binding group, HDACis commonly exhibit a linker connecting the functional group to a capping group (Figure 1a). These two additional features allow medicinal chemists to design an additional pleiotropy of possible inhibitors. We will describe the most important HDACis in the context of preclinical or clinical trials in ALS.

### 3.1 | Treatment with individual inhibitors

HDACis have found broad application in (pre)clinical trials of neurodegenerative diseases (Chuang, Leng, Marinova, Kim, & Chiu, 2009; Echaniz-Laguna et al., 2008). Here, we report (pre)clinical trials of HDACi and their outcome in ALS models. The key characteristics of these studies are summarized in Table 1, while the chemical structures of the reported HDACis are shown in Figure 1.

#### 3.1.1 | Valproic acid and sodium phenylbutyric acid

**Valproic acid** (VPA) or sodium valproate is a short-chain fatty acid (Figure 1b) that is an FDA-approved pan-HDACi and is used for the treatment of bipolar disorders (Cipriani, Reid, Ah, Macritchie, &

Geddes, 2013). Furthermore, it is listed on the “World Health Organization's list of essential medicines.” Sugai and colleagues demonstrated that VPA increased disease duration and lifespan without delaying the disease onset in the SOD1<sup>G93A</sup> mouse model. They also reported neuroprotective effects of VPA against glutamate-induced excitotoxicity in spinal cord cultures (Sugai et al., 2004). Another study investigated long-term treatment with VPA in the SOD1<sup>G93A</sup> mouse model, which slowed down motor neuron death without significantly affecting lifespan (Crochemore et al., 2009). Glutamate-induced excitotoxicity in ALS was also investigated in another study, where rat spinal cord motor neurons were treated with either glutamate or glutamate combined with VPA (Nagańska et al., 2015). VPA-treated motor neurons were preserved, while motor neurons solely treated with glutamate showed signs of apoptotic and autophagic changes. The authors concluded that VPA indeed protects against glutamate-induced neurotoxicity (Nagańska et al., 2015). **Phenylbutyrate** or sodium phenylbutyrate (NaPB), another FDA-approved pan-HDACi, is an aromatic fatty acid (Figure 1b) and is an orphan drug broadly used to treat urea cycle disorders. Phenylbutyrate has extensively been tested and proven to extend the lifespan in ALS disease models, such as the SOD1<sup>G93A</sup> mouse model (Ryu et al., 2005). Recently, NaPB was one of the three major chemical compound hits in a cell-based chemical library screen for modifiers of C9orf72 DPR toxicity of PR<sub>20</sub>. Strikingly, NaPB could also reduce PR<sub>20</sub> toxicity in developing zebrafish embryos (Corman et al., 2019). Both pan-HDACis phenylbutyrate and valproate have been investigated in phase II clinical trials for ALS (see Table 1) but both were unable to prove efficacy (Cudkowicz et al., 2009; Piepers et al., 2009). Valproate-treated subjects did not show a difference in survival or disease progression rate compared to placebo-treated subjects (Cudkowicz et al., 2009). The study investigating NaPB reported a significant increase in blood buffy coat histone acetylation but lacked power to evaluate the efficiency in ALS (Piepers et al., 2009). VPA has poor BBB penetrance, and both VPA and phenylbutyric acid display low inhibitory potency, providing a possible explanation for these findings.

#### 3.1.2 | TSA and Scriptaid

TSA (Figure 1d) is a broad class I and II HDACi, which is a natural product and is effective in multiple cell types. When injected intraperitoneally, it ameliorated motor neuron death and axonal degeneration and it slightly increased the lifespan and delayed disease progression in the SOD1<sup>G93A</sup> mouse model. Additionally, amelioration of atrophy and denervation of the neuromuscular junction was reported and occurred with improved motor functions and reduced gliosis, as well as up-regulation of the **glutamate transporter** in the spinal cord of treated animals (Yoo & Ko, 2011). TSA also improved survival in a mouse model of spinal muscular atrophy, another motor neuron disease (Avila et al., 2007).

**Scriptaid** (Figure 1d) was one of the first HDACis discovered by compound screening approaches and led to the development of vorinostat (or SAHA), which is another FDA-approved HDACi. Scriptaid

**TABLE 1** HDAC inhibitors and their outcome in (pre)clinical trials for ALS

| HDAC inhibitor                    | HDAC target       | Model  | Model organism/stage       | Outcome   |  |
|-----------------------------------|-------------------|--|----------------------------|---|--|
| <i>Preclinical</i>                |                   |  |                            |   |  |
| Trichostatin A                    | Pan-HDAC          | SOD1 <sup>G93A</sup>                         | Mouse model                | Slightly increased lifespan and delayed disease progression (Yoo & Ko, 2011)  |  |
| Scriptaid                         | HDAC1 and HDAC3   | SOD1 <sup>G85R</sup>                         | COS1 cells                 | Inhibited aggresome formation (Corcoran, Mitchison, & Liu, 2004)  |  |
| RGFP966                           | HDAC3             | FUS <sup>R521H</sup>                         | Motor neurons              | Preserved nuclear mutant FUS (Kuta et al., 2020)  |  |
| RGFP109 combined with arimoclomol | HDAC1 and HDAC3   | FUS <sup>R521H</sup>                         | Motor neurons              | Increased nuclear FUS relocalization and effect on DNA repair (Kuta et al., 2020)   |  |
|                                   |                   | SOD1 <sup>G93A</sup>                         |                            | Enhanced HSP induction (Kuta et al., 2020)  |  |
| MC1568                            | HDAC class II     | SOD1 <sup>G93A</sup>                         | Mouse model                | Initial improved motor neuron performance and skeletal muscle electric potential; no duration of effect (Buonvicino et al., 2018)                 |  |
|                                   |                   | SOD1 <sup>G93A</sup>                         | Mouse model                | Restored glutamate uptake capacity in spinal cord; no increased lifespan (Lapucci et al., 2017)   |  |
| ACY-738                           | HDAC6 and class I | Tg FUS <sup>+/+</sup><br>PrP-hFUS-WT3        | Mouse model                | Increased lifespan and delayed disease progression (Rossaert et al., 2019)  |  |
|                                   |                   | FUS <sup>P525L</sup><br>FUS <sup>R521H</sup> | iPSC-derived motor neurons | Reversed axonal transport deficits and increased $\alpha$ -tubulin acetylation (Guo et al., 2017)   |  |
| Tubastatin A                      | HDAC6             | FUS <sup>P525L</sup><br>FUS <sup>R521H</sup> | iPSC-derived motor neurons | Reversed axonal transport deficits and increased $\alpha$ -tubulin acetylation (Guo et al., 2017)   |  |
|                                   |                   | RAPGEF2 <sup>E1357K</sup>                    | Patient fibroblasts        | Rescue of abnormal mitochondrial network (Heo et al., 2018)   |  |
|                                   |                   | FUS <sup>R521H</sup>                         | Motor neurons              | Effect on DNA repair (Kuta et al., 2020)  |  |
| Valproic acid                     | Pan-HDAC          | SOD1 <sup>G93A</sup>                         | Mouse model                | Slowed down motor neuron death but no increased lifespan (Crochemore et al., 2009)  |  |
|                                   |                   | SOD1 <sup>G93A</sup>                         | Mouse model                | Increased disease duration and lifespan but no delayed disease onset (Sugai et al., 2004)   |  |
|                                   |                   | SOD1 <sup>G86R</sup>                         | Mouse model                | Slight, not significant delayed disease onset (Rouaux et al., 2007)   |  |
|                                   |                   | Glutamate excitotoxicity                     | Rat motor neurons          | Preserved motor neuron culture (Nagańska, Matyja, Taraszewska, & Rafałowska, 2015)  |  |
|                                   |                   | Glutamate excitotoxicity                     | Mouse spinal cord culture  | Preserved motor neuron culture (Sugai et al., 2004)   |  |
|                                   |                   | combined with lithium                        | SOD1 <sup>G93A</sup>       | Mouse model   | Increased lifespan and delayed disease onset (Feng et al., 2008) |
|                                   |                   | Entinostat combined with resveratrol         | HDAC1–3 Sirt1 activator    | SOD1 <sup>G93A</sup>  | Mouse model  |
| Resveratrol                       | Sirt1 activator   | SOD1 <sup>G93A</sup> mouse model             | Mouse model                | Increased motor neuron function and extended survival (Lee et al., 2012; Mancuso et al., 2014)  |  |
|                                   |                   | hSOD1 <sup>G93A</sup>                        | Motor neuron-like cells    | No effects (Markert et al., 2010)<br>Neuroprotective via up-regulation of SIRT1 hSOD1 <sup>G93A</sup> motor neuron-like cells (Wang et al., 2011) |  |

**TABLE 1** (Continued)

| HDAC inhibitor                                      | HDAC target                              | Model  | Model organism/stage                              | Outcome   |
|---|--|--|---|---|
| Vorinostat combined with arimoclolomol              | Pan-HDAC                                 | FUS <sup>R521H</sup>   | Motor neurons                                     | Increased nuclear FUS relocalization and effect on DNA repair (Kuta et al., 2020)   |
|   |  | SOD1 <sup>G93A</sup>   |   | Enhanced HSP induction (Kuta et al., 2020)  |
| Sodium phenylbutyrate                               | Pan-HDAC                                 | SOD1 <sup>G93A</sup>   | Mouse model                                       | Increased lifespan (Ryu et al., 2005)   |
|   |  | SOD1 <sup>G93A</sup>   | Mouse model                                       | Increased lifespan (Del Signore et al., 2009)   |
|   |  | SOD1 <sup>G93A</sup><br>C9orf72<br>PR <sub>20</sub> toxicity | Mouse model<br>U2OS and NSC-34 cells<br>Zebrafish | Increased lifespan (Petri et al., 2006)<br>Reduced toxicity and increased survival of developing zebrafish (Corman et al., 2019)              |
| combined with AEOL10150<br>combined with riluzole   | Antioxidant                              | SOD1 <sup>G93A</sup>   | Mouse model                                       | Increased lifespan (Petri et al., 2006)   |
|   |  | SOD1 <sup>G93A</sup>   | Mouse model                                       | Increased lifespan (Del Signore et al., 2009)   |
| Selisistat  | Sirt1                                    | SOD1 <sup>G93A</sup>   | SH-SY5Y cells                                     | Restore viability in cells infected with mutant SOD1 <sup>G93A</sup> but not via Sirt1 or Sirt2 inhibition (Valle et al., 2014)               |
| <i>Live imaging</i>                                 |  |  |   |   |
| [ <sup>11</sup> C]Martinostat                       | Pan-HDAC (1–3 and 6)                     | ALS patients versus healthy controls                         |   | No significant differences in [ <sup>11</sup> C] Martinostat density in vivo or HDAC expression levels (protein and mRNA) (Dios et al., 2019) |
| <i>Clinical</i>                                     |  |  |   |   |
| Valproic acid                                       | Pan-HDAC (classes I and II)              |  | Human phase II                                    | No difference in survival compared to placebo (Piepers et al., 2009)  |
| Sodium phenylbutyrate                               | Pan-HDAC (classes I and II)              |  | Human phase II                                    | Safe and tolerable; no efficacy detected (Cudkowicz et al., 2009)   |
| Sodium phenylbutyrate combined with AMX0035 (TUDCA) | Pan-HDAC (classes I and II)<br>Bile acid |  | Human phase II                                    | Ongoing NCT03127514   |

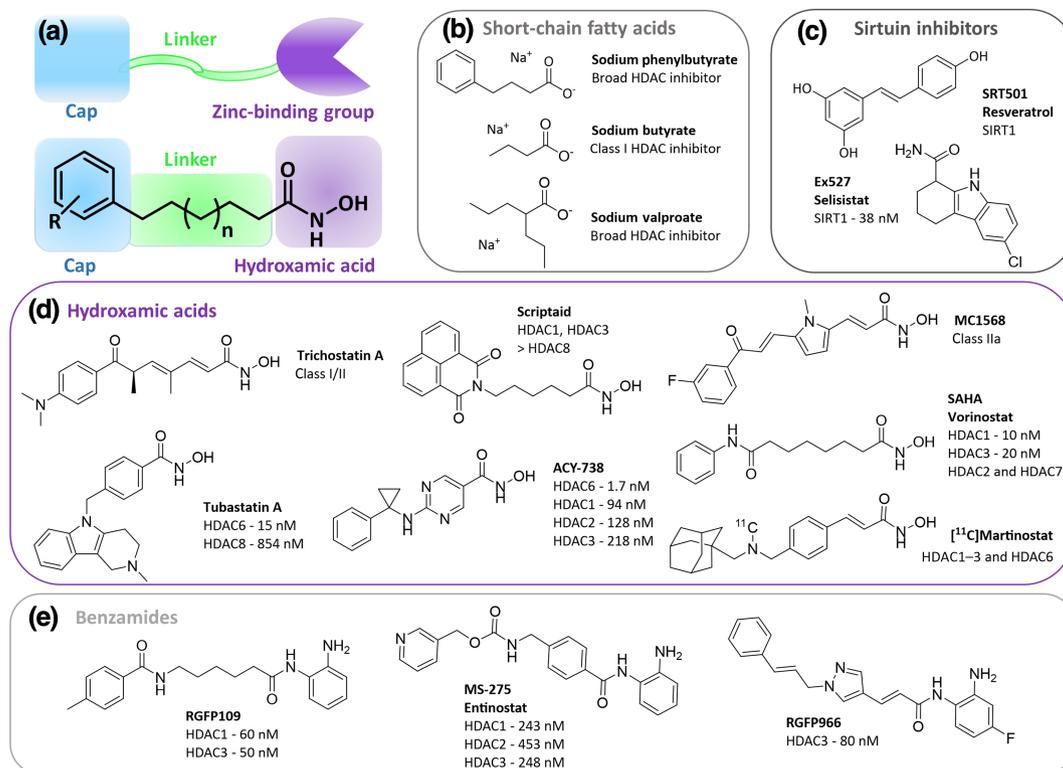
inhibited aggresome formation in a 20,000-compound screening approach in a SOD1<sup>G85R</sup> COS1 cell model (Corcoran et al., 2004), highlighting a potential link between ALS and HDAC activity.

### 3.1.3 | Tubastatin A and ACY-738

**Tubastatin A** is an HDAC6-selective hydroxamic acid-based inhibitor (Figure 1d) (Butler et al., 2010). Tubastatin A displays high HDAC6 selectivity due to its phenylhydroxamic acid core. It was developed based on the HDAC6-selective inhibitor tubacin, which is used rather as a research tool than a drug because of its lipophilicity and size (Butler et al., 2010). **ACY-738** was developed by Acetylon Pharmaceuticals and displays improved BBB penetrance and HDAC6 selectivity but also inhibits HDACs 1, 2, and 3 (Jochems et al., 2014). Recently, a review on HDAC6-selective inhibitors in neurodegenerative diseases was published (Shen & Kozikowski, 2020). We demonstrated that HDAC6 inhibition reversed axonal transport defects in patient-derived iPSC motor neurons (FUS<sup>P525L</sup> and

FUS<sup>R521H</sup> mutations). We tested ACY-738 and tubastatin A in both FUS lines, reversing the axonal transport defects (Guo et al., 2017). In line with these findings, another study showed that HDAC6 inhibition with tubastatin A stabilized the microtubule network. It rescued intracellular distribution defects of mitochondria and **BAX** in fibroblasts from an early-onset sALS patient with a novel RAPGEF2<sup>E1357K</sup> mutation (Heo et al., 2018).

In another study, we treated an ALS transgenic mouse model (Tg FUS+/+) with ACY-738 and found that ACY-738 restored histone hypoacetylation levels, slowed down the disease progression, and extended the lifespan by 68% (Rossaert et al., 2019). This hypoacetylation was not caused by a differential expression of HDACs but rather by increased HDAC activity. Crossbreeding the transgenic FUS mice with *Hdac6* knockout mice did not increase the lifespan, implying that the treatment effect was not HDAC6-mediated but, instead, dependent on class I HDACs. Additionally, the mouse model showed metabolic dysregulation already before phenotype onset, and ACY-738 treatment reversed this dysregulation. Transcriptome and proteome analysis on spinal cord tissue revealed that genes and proteins



**FIGURE 1** HDAC inhibitors for ALS which are covered in this review. (a) Linker design of general HDAC inhibitors and illustration of the hydroxamic acid-based design. (b–e) Structures of HDAC inhibitors in (pre)clinical trials for their therapeutic potential in ALS such as short-chain fatty acids HDAC inhibitors (b), sirtuin inhibitors (c), hydroxamic acid-based HDAC inhibitors (d), and benzamide-based HDAC inhibitors (e)

associated with fatty acid and cholesterol biosynthesis as well as beta-oxidation were down-regulated while glycolysis, pentose phosphate pathway, and lipid transport were up-regulated. These defects were restored by ACY-738 treatment, implying an association between histone hypoacetylation and metabolic dysregulation in FUS-mediated ALS pathologies (Rossaert et al., 2019).

### 3.1.4 | MC1568

**MC1568** is a selective class II HDACi that is a hydroxamic acid based on an aryloxopropenylpyrrolyl-based linker, rendering it selective for class II HDACs (Figure 1d) (Mai et al., 2005). The glial **EAAT2** glutamate transporter, whose reduced expression was suggested to cause motor neuron degeneration, was considered as a potential target counteracting excitotoxicity-based mechanisms in ALS (Lapucci et al., 2017). Treatment with MC1568 induced EAAT2 expression in mouse glial cells. To investigate the role of EAAT2 in ALS, expression levels in the SOD1<sup>G93A</sup> mouse model were monitored and mRNA levels were unaltered while protein expression was significantly reduced at a late stage of the disease. Treatment with MC1568 fully restored EAAT2 protein levels in spinal cord as well as glutamate uptake capacity. However, treatment did not prolong lifespan, indicating that restoration of glutamate uptake, alone, was not sufficient to obtain a therapeutic effect (Lapucci et al., 2017). In another study, a significant increase of class II HDAC mRNAs (HDACs

4, 5, and 6) was detected in skeletal muscle at later stages of the disease, but not in motor neurons of SOD1<sup>G93A</sup> mice. MC1568 treatment improved motor performance during the first phase of the disease but failed to improve worsening of motor performance at later stages, and it did not affect survival. Improved motor performance did not correlate to reduced motor neuron degeneration. Moreover, increased muscle electrical potentials, an impaired activation of MIR206/FGFBP-1-dependent muscle re-innervation signalling, and an increased expression of myogenic genes in muscle were observed. These differences did not persist and almost paralleled to untreated conditions, indicating that MC1568 was not a favourable HDACi because of the failure to improve the phenotype in the long term (Buonvicino et al., 2018).

### 3.1.5 | Selisistat and resveratrol

By treating mutant SOD1<sup>G93A</sup> cells with different SIRT inhibitors and a SIRT1 activator, only neuroprotective effects were found by treatment with the SIRT1 inhibitor **selisistat** (Ex527) (Figure 1c) (Valle et al., 2014). However, it could not be proven that the effect was a result of SIRT1 inhibition, suggesting that SIRT inhibitors could display unknown off-target effects (Valle et al., 2014).

**Resveratrol** (Figure 1c) regulated SIRT1 activity and improved motor function and survival in the SOD1<sup>G93A</sup> mouse model by modulating p53 acetylation (Lee et al., 2012). In a cell-based model,

resveratrol was neuroprotective by up-regulating SIRT1 expression in mutant hSOD1<sup>G93A</sup> motor neuron-like cells (Wang et al., 2011). In the SOD1<sup>G93A</sup> model, resveratrol inhibited apoptosis of motor neurons and extended the lifespan through multiple pathways including SIRT1 and AMPK activation, stabilization of autophagy, suppression of oxidative stress, and acetylation of p53 (Lee et al., 2012; Mancuso et al., 2014).

### 3.2 | Combination therapies using HDACis

Exerting neuroprotective properties, HDAC inhibition could be a promising target in neurodegenerative disorders, including ALS (Chuang et al., 2009; Gal et al., 2013). However, major concerns include toxic side effects, low inhibition selectivity, and specificity as well as efficacy. Therefore, different combinational therapies to decrease potentially toxic or harmful doses, as well as side effects and off-target effects, have been investigated so far. Next, we will give an overview of recent combinational studies including HDACi.

#### 3.2.1 | VPA and lithium

Due to their neuroprotective effects, valproate and lithium are both compounds that are used to treat bipolar disorders. In a short communication, Feng and colleagues demonstrated a synergistic effect in delaying disease onset and prolongation of lifespan in the SOD1<sup>G93A</sup> mouse model. The combinational therapy showed a greater effect than treatment with one drug alone, and the authors correlated their neuroprotective findings to improved GSK-3 $\beta$ -mediated serine phosphorylation (Feng et al., 2008).

#### 3.2.2 | Entinostat and resveratrol

Entinostat (MS-275) is a benzamide-based HDAC 1 and 3 inhibitor with anti-tumour efficacy (Figure 1e) (Saito et al., 1999). A proof-of-concept study was recently published, reporting a combination therapy of resveratrol and entinostat (Schiaffino et al., 2018). A delay of 20 days in the disease onset and a 12% increase of lifespan were observed in the SOD1<sup>G93A</sup> mouse model, using the SIRT1 activator and the pan-HDACi (Schiaffino et al., 2018). Two different doses were tested, and the higher dose was a 50-fold lower entinostat concentration than the one used in a neuroprotection study of brain ischaemia (Murphy et al., 2014). Resveratrol alone also improved motor neuron function and increased the lifespan of the SOD1<sup>G93A</sup> mice (Lee et al., 2012; Mancuso et al., 2014). An increased number of motor neurons accompanied the increased SIRT1 and BDNF expression in the spinal cord of the treated mice. However, others could not confirm this positive effect of resveratrol in the SOD1<sup>G93A</sup> model (Markert et al., 2010). HDACs are known to deacetylate the transcription factor NF- $\kappa$ B, which is involved in regulating differentiation or cell death (Chen & Greene, 2004). NF- $\kappa$ B and its subunit RelA are modified by acetylation, and deacetylation promotes nuclear export of NF- $\kappa$ B and thus terminating the transcriptional

response (Chen & Greene, 2004). Entinostat in combination with resveratrol administration restored the acetylation state of NF- $\kappa$ B RelA in the spinal cord, which could be responsible for delaying disease onset and increasing the lifespan in the SOD1<sup>G93A</sup> mouse model (Schiaffino et al., 2018).

#### 3.2.3 | Vorinostat or RGFP109 and arimoclomol

Vorinostat (SAHA) has been approved for the treatment of cutaneous T-cell lymphoma, and according to *clinicaltrials.gov*, 315 studies use vorinostat (Figure 1d). It had been shown earlier that FUS<sup>R521H</sup> motor neurons exhibited impaired histone acetylation, which could be reversed upon treatment with vorinostat (Tibshirani et al., 2015). Recently, Kuta et al. (2020) investigated the effects of different HDACis on co-inducing the heat shock response via HSPs in motor neurons. Motor neurons are relatively unresponsive to induction of a heat shock response, and up-regulation of this stress pathway might be neuroprotective by chaperoning misfolded proteins by HSPs (Batulan et al., 2006). HSP90 inhibition by the chemical compound NXD30001 induces HSPs and especially HSP70 up-regulation, via HSF1. Vorinostat and NXD30001 co-treatment increased HSP70 expression upon HSP90 inhibition (Cha et al., 2014). In the study of Kuta et al. (2020), it was discovered that HDAC inhibition could enhance co-induction of HSP70 by the HSP inducer arimoclomol in different stress scenarios. HDAC inhibition could preserve DNA repair and promote nuclear retention of mutant FUS through non-HSP-mediated mechanisms. Both vorinostat and RGFP109, another benzamide-derived HDAC1- and HDAC3-selective inhibitor (Figure 1e), in combination with arimoclomol were able to induce HSP70 expression in SOD1<sup>G93A</sup> motor neurons. Additionally, they were able to increase nuclear FUS and elevate DNA repair mechanisms in the FUS<sup>R521H</sup> motor neurons. In summary, these results strongly suggest that these effects are due to multiple mechanisms of neuroprotection by both HDAC-inhibiting drugs and arimoclomol (Kuta et al., 2020).

Arimoclomol is currently in a phase II/III clinical trial for ALS (NCT00706147) and in inclusion body myositis and has a favourable safety profile (Lanka, Wieland, Barber, & Cudkowicz, 2009). This drug is believed to work via activation of molecular chaperones, such as the heat shock response, to clear aggregates. The study was completed, and arimoclomol was being well tolerated. Noteworthy is that it is the first study targeting a genotypically homogenous ALS population exhibiting SOD1 mutations (Benatar et al., 2018). A phase III ALS study is currently ongoing (NCT03491462).

#### 3.2.4 | NaPB and riluzole or AEOL 10150

NaPB in combination with riluzole or the antioxidant AEOL 10150 extended survival in the SOD1<sup>G93A</sup> model (Del Signore et al., 2009; Petri et al., 2006). NaPB is currently investigated in an ongoing clinical phase II trial in combination with tauroursodeoxycholic acid

(TUDCA), named AMX0035 (NCT03127514). TUDCA is a bile acid and is thought to prevent apoptosis, to be cytoprotective, and to act as a chemical chaperone. A first study in a small number of patients ( $n = 34$ ) provided preliminary clinical data indicating that TUDCA might be effective in ALS (Elia et al., 2016).

#### 4 | IMAGING STUDIES OF HDACs

Ample evidence links decreased histone deacetylation to neurodegeneration (Rouaux et al., 2003; Valle et al., 2014). In line with this, histone hypoacetylation was an associated feature in ALS models (Bennett et al., 2019; Del Signore et al., 2009; Ryu et al., 2005). However, these findings were not confirmed in an in vivo brain imaging study with a pan-HDAC PET imaging ligand reported by Dios et al. (2019). To analyse the potential of HDACs as biomarkers, the authors investigated class I and II HDAC protein and transcription levels in post-mortem motor cortex and spinal cord samples, combining this with an in vivo [ $^{11}\text{C}$ ]Martinostat (Figure 1d) study, comparing ALS patients and healthy controls. [ $^{11}\text{C}$ ]Martinostat is a novel HDAC PET ligand to assess in vivo brain HDAC alterations (Wang et al., 2014). No significant differences in HDAC levels measured by either Western blot or PCR, or in [ $^{11}\text{C}$ ]Martinostat-PET uptake were observed in ALS patients, compared to controls. It was concluded that HDAC isoform alterations may not be a dominant pathological feature at bulk tissue levels in ALS (Dios et al., 2019). This is in contrast to earlier findings of other groups reporting HDAC differences on protein or mRNA expression levels (Janssen et al., 2010; Körner et al., 2013). This discrepancy can be due to many reasons. HDAC expression was altered throughout both normal ageing and during disease progress, making it difficult to assess the exact relevance of differences (Gilbert et al., 2019; Valle et al., 2014). Furthermore, the imaging study did not differentiate between ALS types in the post-mortem or in the in vivo part of the study (familial vs. sporadic, as well as genetic background). The authors suggested that there might be alterations due to disease subgroups of ALS. Research based on ALS subtype could be an intriguing approach for further studies, as HDAC levels may vary depending on the mutation and thus associated pathways. Furthermore, the authors did not investigate mRNA differences in spinal cord, in which differences in HDAC levels were previously shown (Janssen et al., 2010). It would be very interesting to investigate HDAC distribution over the spinal cord or in other tissues of patients. The recently developed HDAC6-selective PET radiotracer [ $^{18}\text{F}$ ]EKZ-001 ([ $^{18}\text{F}$ ]Bavarostat), the [ $^{18}\text{F}$ ]- and [ $^{11}\text{C}$ ]Tubastatin-derived PET radiotracers, and the class IIa HDAC-selective radiotracer [ $^{18}\text{F}$ ]TFAHA might provide excellent tools to study HDACs selectively in different diseases (Celen et al., 2020; Laws et al., 2019; Lu et al., 2016; Strebl et al., 2017; Tago, Toyohara, & Ishii, 2020; Vermeulen, Ahamed, Luyten, & Bormans, 2019). Of particular interest for future research is the just recently published study by Koole and colleagues, investigating tolerability in a first-in-man study of [ $^{18}\text{F}$ ]EKZ-001. The HDAC6 radiotracer was found to be safe and well tolerated by healthy adult subjects and showed HDAC6

distribution with highest tracer uptake in the hippocampus and entorhinal cortex. This provides an exciting platform for future research and HDAC6-based therapies (Koole et al., 2020). For further reading on PET imaging in ALS, we refer to the review by Swinnen and colleagues (2016).

Taken together, all these data strongly suggest that further clinical and preclinical imaging studies may deepen our understanding of the involvement of HDACs or other proteins in the progress, severity, and mechanisms of ALS.

#### 5 | HATs, HDACs, OR PROTEINS REGULATING THEIR ACTIVITY AS THERAPEUTIC OPPORTUNITIES

The question remains which mechanisms and proteins are involved in and are responsible for the hypoacetylation found in neurodegeneration. There are several possibilities at both the HDAC and HAT levels to achieve this. On the one hand, either HDACs may be overexpressed or HDAC activity is up-regulated. For example, this could be regulated by the recruitment of other proteins. On the other hand, it is also possible that the counteracting HATs are affected via either differential expression or reduced activity.

It is indeed important to unravel imbalances in the equilibrium between HDACs and HATs. Investigating changes in HAT activity and expression levels, Rouaux et al. (2003) found a decrease of the HAT CBP in lumbar spinal cord of SOD1<sup>G86R</sup> mice. This was associated with decreased acetylation levels of histone H3 in the nuclei of motor neurons (Rouaux et al., 2003). Interestingly, the HAT ELP3 acetylates histones H3 and H4 and thus directly regulates HSP70 expression in yeast (Han et al., 2008). When knocked down, it caused motor axonal abnormalities in zebrafish embryos (Simpson et al., 2009). ELP3 defects may be involved in motor neuron degeneration through impaired transcription of HSP70 (Alao, 2007).

One key hallmark may be changes in activity of both HDACs and HATs, regulated by their associated protein complexes. The assembly or disassembly of these big protein complexes, such as the Sin3a, CoREST, or NuRD complexes, can regulate the activity of HDACs. In line with this, deletion of Set3, which is a yeast HDAC complex member and homologous to the human ASH1, suppressed TDP-43 toxicity (Armakola et al., 2012).

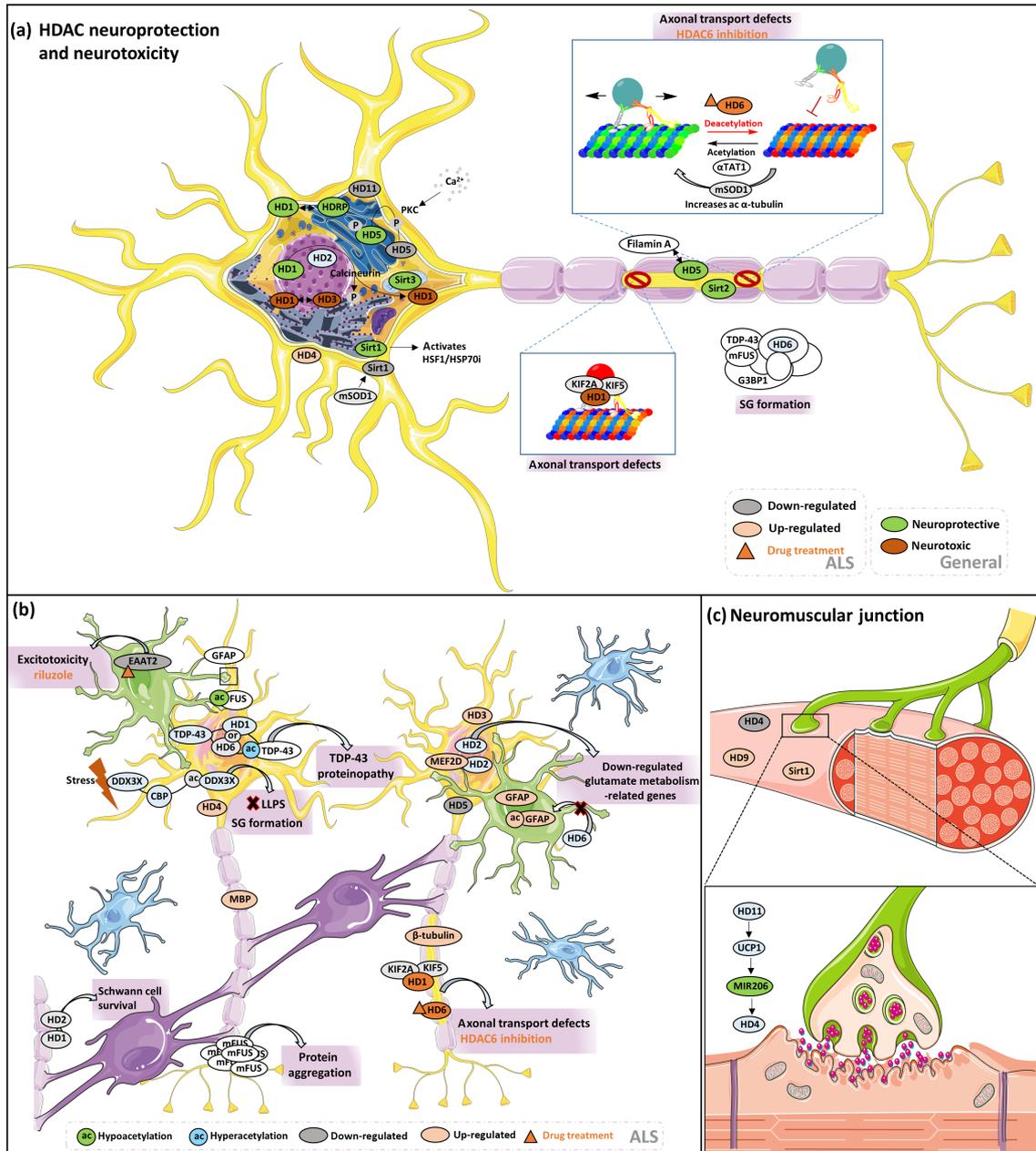
FUS directly interacts with HDAC1 to regulate DNA damage response and repair in neurons and also directly binds and inhibits the HAT activities of CBP/p300 (Wang et al., 2013; Wang et al., 2008). Association with CBP/p300 happens through the N-terminal domain of FUS, which leads to inhibition of CCND1 transcription following DNA damage (Wang et al., 2008). Interestingly, the glycine-rich and C-terminal domains of FUS, which harbour the majority of familial ALS mutations, resemble the two domains necessary for HDAC1 interaction (Qiu et al., 2014; Wang et al., 2013). The RNA-binding protein RBM45 competed with HDAC1 for binding to FUS, thereby regulating the recruitment of HDAC1 to DNA damage sites (Gong et al., 2017). When FUS contained the fALS-associated R521C

mutation, it interacted with RBM45 rather than with HDAC1, suggesting that RBM45 is a key regulator in FUS-related DNA damage response signalling (Gong et al., 2017). Alternatively, shifts in activity could also result from HATs or HDACs being dysregulated through post-translational modification.

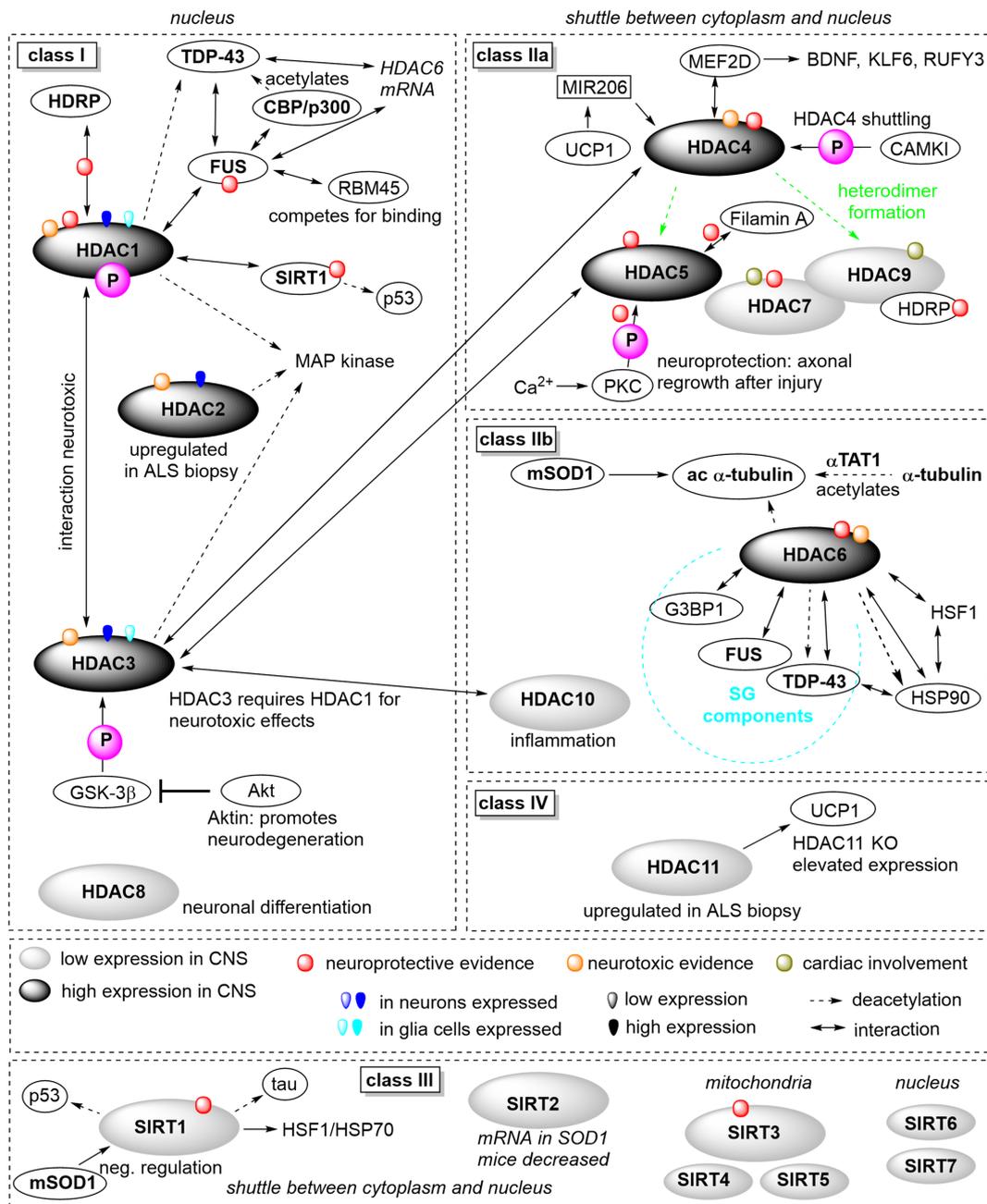
It is also important to further elucidate the cell types contributing to ALS and how HDACs are involved in this. Liu et al. (2013) investigated post-mortem ALS spinal cord proteins using proteomics and immunoprecipitation approaches and detected an increase of glial fibrillary acidic protein (GFAP) in both insoluble and soluble fractions. Also, acetylated GFAP was enriched in the soluble fraction in addition

to  $\beta$ -tubulin and myelin basic protein (MBP). GFAP plays an important role in astrocyte–neuron interaction and cell–cell communication. This study points towards astrocytes being involved in the disease, and the authors conclude that GFAP acetylation could be a result of impaired HDAC6 activity (Liu et al., 2013).

Of particular interest are the findings of Chen et al. (2018) characterizing the global histone acetylation levels in yeast ALS proteinopathy models of FUS and TDP-43. A distinctive acetylation pattern for each mutation was found. While FUS overexpression caused histone hypoacetylation, resulting in a global decrease in gene transcription, TDP-43 on the contrary caused



**FIGURE 2** HDAC mechanisms related to the pathology of ALS. (a) Up- and down-regulation differences found in ALS and general neuroprotective and neurotoxic mechanisms. (b) Variety of neuronal and non-neuronal cells and ALS mechanisms involving HDACs and associated proteins. (c) The neuromuscular junctions and mechanisms indicating HDACs in ALS. HD, HDAC. Cell illustrations are modified from Smart Servier Medical Art licenced under a Creative Commons Attribution 3.0 Unported License (<https://creativecommons.org/licenses/by/3.0/>)



**FIGURE 3** Interaction pathways and biochemical mechanisms associated with HDAC classes I–IV, individual HDACs, and interaction partners, expression patterns, and substrates in the context of neurodegeneration and ALS

modest hyperacetylation, suggesting enhanced gene transcription. Remarkably, the hypoacetylation caused by FUS was only found on lysine14 of histone H3, indicating specific histone H3 deacetylation sites. It is surprising that the findings suggest that TDP-43 and FUS could lead via different pathways to ALS (Bennett et al., 2019; Chen et al., 2018).

A summary of the findings, reported here, in a global ALS setting can be found in Figure 2, and the complex HDAC networks within cells are shown in Figure 3. We hope these overviews help to elucidate the challenges of targeting neurotoxic mechanisms, without hampering neuroprotective features of the HDAC superfamily.

## 6 | CONCLUSIONS

We have discussed the opposing roles for individual HDACs in relation to ALS, which possibly complicate the translation of HDAC inhibition into a therapeutic strategy. Recent research suggested that this may originate from differential expression of HDACs during a specific disease stage and within a specific temporal window. In addition, this may depend on tissue or cell type as well as on different mechanisms at play, such as protein–protein interactions, cellular localization, post-translation modifications, or alternative splicing (Thomas & D'Mello, 2018).

While the activity of some HDACs may contribute to ALS pathogenesis, other isoforms play beneficial homeostatic roles. Thus, it is necessary to carefully fine-tune the potential therapeutic agents towards selectivity, rather than using pan-HDACis. The doses administered for selective and specific *in vivo* targeting are crucial, as overdosing will probably lead to off-target effects, while too low doses may not show any efficacy. Therefore, research needs to further focus on unravelling the *in vivo* targets of HDACis in order to rule out side effects or off-target effects. This should be investigated not only in disease models but especially also in humans. PET imaging studies will provide a platform to evaluate target engagement, BBB penetrance, molecular activity, and optimal dosage of potential drugs.

The majority of conducted research used the mutant SOD1 mouse model for validation of potential HDACis. Other models should be employed to reproduce findings. All studies that showed promise in the SOD1 mouse models subsequently failed to show efficacy in clinical trial settings. Therefore, it is of utmost importance to investigate the role of neuroprotection and toxicity of each HDAC while taking into consideration different cell and tissue regions, sporadic versus familial, age, and disease stage differences in ALS. In conclusion, we are far away from relieving the devastating burdens of patients diagnosed with ALS. However, we and others have succeeded in showing effects of HDACis in modulating some relevant ALS phenotypes (Guo et al., 2017; Kuta et al., 2020; Rossaert et al., 2019). Hopefully, this and further research can provide better insights which, hopefully, will lead to new therapeutic approaches for neurodegenerative disorders.

## Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (<http://www.guidetopharmacology.org>) and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Fabbro et al., 2019; Alexander, Kelly et al., 2019a, b; Alexander, Mathie et al., 2019).

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ORCID

Yvonne E. Klingl  <https://orcid.org/0000-0003-2779-3357>

Donya Pakravan  <https://orcid.org/0000-0002-3165-984X>

Ludo Van Den Bosch  <https://orcid.org/0000-0003-0104-4067>

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