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Positron Emission Tomography in Amyotrophic Lateral Sclerosis: Towards Targeting of Molecular Pathological Hallmarks

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Abstract

The past decades, extensive efforts have been made to expand knowledge on Amyotrophic Lateral Sclerosis (ALS). However, clinical translation of this research in terms of earlier diagnosis and improved therapy, remains challenging. Since more than 30% of motor neurons are lost when symptoms become clinically apparent, techniques allowing non-invasive, in vivo detection of motor neuron degeneration are needed in the early, pre-symptomatic disease stage. Furthermore, it has become apparent that non-motor signs play an important role in the disease and there is an overlap with cognitive disorders, such as frontotemporal dementia (FTD). Radionuclide imaging, such as positron emission tomography (PET) and single photon emission computed tomography (SPECT), form an attractive approach to quantitatively monitor the ongoing neurodegenerative processes. Although [^{18}F]-FDG has been recently proposed as a potential biomarker for ALS, active targeting of the underlying pathologic molecular processes is likely to unravel further valuable disease information and may help to decipher the pathogenesis of ALS. In this review we provide an overview of radiotracers that have already been applied in ALS and discuss possible novel targets for in vivo imaging of various pathogenic processes underlying ALS onset and progression.

Key words: ALS, neurodegeneration, PET, targets, radiotracers

Introduction

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's or Charcot's disease, is a devastating and fatal adult-onset neurodegenerative disorder characterized by progressive degeneration of both upper motor neurons (UMN) in the motor cortex and lower motor neurons (LMN) in the spinal cord and brainstem. This ensuing motor neuron loss results in fasciculation's, muscle wasting, weakness, spasticity and paralysis. Eventually, respiratory muscle weakness results in fatal respiratory failure in most ALS patients. ALS is part of a motor neuron disease spectrum also containing primary lateral sclerosis (PLS), showing exclusively UMN involvement, and progressive muscular atrophy (PMA), showing exclusively LMN involvement; both may progress to ALS in later disease stages [1-3].

Although ALS was already discovered as disease entity over 150 years ago, many questions regarding the cause, onset, non-motor impact and progression of the disease remain unanswered [4]. The median survival of ALS patients still does not exceed 36 months and the absence of a suitable disease-modifying approach or neuroprotective drug, is an important hurdle in ALS management [5]. Despite the large number of clinical trials testing varieties of possible therapeutic agents, Riluzole (2-amino-6-(trifluoromethoxy) benzothiazole) remains the only FDA approved drug for ALS. Riluzole is an anti-glutamatergic drug that suppresses glutamate release, prevents glutamate receptor hypofunction and stimulates glutamate uptake by activation of glutamate transporters [6-8]. Unfortunately, this glutamate-modulating agent increases overall survival with only 3 to 4 months [9, 10]. Given the multisystem involvement in ALS pathogenesis, the interest in drug cocktails, acting on a combination of disease-regulating targets, is rising [11].

Of all ALS cases, only 5-10% is familial (fALS), mostly showing dominant Mendelian inheritance patterns [12], while the majority are sporadic cases (sALS). Several gene mutations, such as mutations in superoxide dismutase 1 (SOD1) located on chromosome 21 and responsible for 20% of fALS cases [13], fused in sarcoma (FUS) located on chromosome 16 and responsible for 3-5% of fALS cases) [14, 15], and TAR-DNA binding protein (TDP-43) located on chromosome 1 and responsible for 5% of fALS cases [16]; were identified as causative for ALS. However, the gene defect currently recognized as the major genetic cause of ALS (40% of fALS cases) is a hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 gene (*C9orf72*). Patients harboring this repeat usually present younger, show poorer survival and typically have a strong family history of neurodegenerative disease [17-19]. Several of these fALS causing gene mutations can also cause frontotemporal dementia (FTD). Furthermore, it has been shown that the majority of ALS cases and a subset of FTD cases share pathophysiological characteristics with aggregation of TDP-43 [20-22], and about 15% of all ALS patients have clinically

apparent cognitive impairment [23, 24], suggesting that both disorders form a clinical continuum. Recently, Canosa et al. showed significant differences in frontal and prefrontal glucose metabolism in patients with stand-alone ALS when compared to patients with co-morbid FTD using [^{18}F]-FDG PET [25]. Interestingly, patients with milder cognitive impairment showed less explicit frontal hypometabolism. These results confirm the hypothesis of a clinical continuum between both disorders and indicate that [^{18}F]-FDG PET could be applied to determine the cognitive state of ALS patients. The remaining 90-95% of all ALS cases are sporadic (sALS). Although the presence of fALS causing gene mutations has been shown in some sALS cases and the existence of susceptibility genes in sALS has been suggested, the primary cause of the majority of ALS cases remains unraveled.

In analogy with many other major neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (PD), the pathophysiology of ALS is considered a complex, multifactorial interaction of genetic, molecular and cellular mechanisms [26, 27]. The main pathologic processes take place in the motor neuron cell bodies, the axons and at the neuromuscular junction and in the surrounding cells. Among the long list of possibly involved pathogenic pathways, several mechanisms are thought to be key players in the relentless degeneration of motor neurons. Oxidative stress, resulting from increased generation and accumulation of reactive oxygen species (ROS), is present in cerebrospinal fluid (CSF) and serum of ALS patients [28-30] which provides evidence for neurodegeneration due to oxidative damage. Mitochondrial DNA in the spinal cord of ALS patients shows elevated mutation rates [31] and these abnormalities are already present before symptom onset [32]. Furthermore, mutated TDP-43 and SOD1 can migrate to mitochondria and induce mitochondrial dysfunction as well [33, 34]. The role of glutamate-mediated excitotoxicity and injury due to overstimulation by excitatory mediators, was suggested in ALS pathogenesis by detection of high glutamate levels in CSF [35, 36]. The positive effect of the anti-glutamatergic drug Riluzole also supports the role of excitotoxicity in ALS pathogenesis. Protein-aggregates, a generally accepted pathological hallmark of neurodegenerative diseases, are mainly found in spinal motor neurons in ALS and can consist of cytostatin C, transferrin [37], neuro-filament rich hyaline aggregates [38, 39] or TDP-43 [40, 41]. Interestingly, spreading of these TDP-43 containing inclusions was shown to be associated with disease progression through the neuropathological disease stages [42, 43]. Another important hallmark is chronic neuroinflammation, characterized by activation of microglia and astrocytes, a feature shown to correlate with disease progression [44, 45]. These pathways offer potentially promising targets for non-invasive in vivo imaging of motor neuron degeneration in ALS, and may form targets for novel therapies, for which imaging can lead to proof-of-principle.

Additive value of in vivo imaging in ALS

Since there is no diagnostic test with sufficient specificity and sensitivity available for ALS, diagnosis predominantly relies on the presentation of clinical symptoms listed in the revised El Escorial [46] and Awaji-Shima [47] criteria, supported by electrophysiological and genetic testing and sometimes neuroimaging. Nevertheless, the diagnostic process is associated with a significant uncertainty level due to limited sensitivity of the applied criteria, often resulting in diagnostic delay (up to 1 year) [48]. Incorporation of non-invasive in vivo imaging techniques, such as Positron Emission Tomography (PET), Single Photon Emission Computed Tomography (SPECT) and Magnetic Resonance Imaging (MRI), into the standard diagnostic regimen, might provide crucial additional and complementary disease information and may therefore increase the confidence level and the extent of early diagnosis [49].

Currently, the main role of neuroimaging remains limited to excluding structural pathologies which can cause symptoms similar to those of ALS, such as Kennedy's disease and X-linked spino-bulbar muscular atrophy, which may clinically present as ALS. Here, structural MRI is the primary investigation of choice. In two structural T2-weighed imaging studies however, this technique appeared to be neither sufficiently sensitive (76% and 90%, respectively) nor specific (75% and 21%, respectively) as a diagnostic tool [50, 51]. Although more advanced MR techniques, such as diffusion tensor imaging (DTI) [52-54], voxel-based morphometry (VBM) [55] and resting state functional MRI (rs-fMRI) [56-58] have shown promising results and might be useful as non-invasive diagnostic markers, their true diagnostic potential remains to be elucidated. In 2010 however, Filippini et al. reported that multimodal MRI, combining fractional anisotropy, grey matter and radial diffusity measures, could discriminate ALS patients from healthy controls with an accuracy of 90%, highlighting the potential of multimodel MRI as a diagnostic tool for ALS [59].

Radionuclide imaging techniques, such as PET and SPECT, may play a new role in earlier and more accurate ALS diagnosis and disease monitoring [49, 60]. Targeting (via receptors, transporters, deposition proteins etc.) of key cellular pathways involved in ALS pathogenesis may visualize and quantify these ongoing processes, providing important additional information and allowing disease staging. Since the underlying pathologic processes might be ongoing long before symptom onset, these tracers might allow earlier (pre-symptomatic) diagnosis. Taken together, non-invasive in vivo imaging of the pathological disease hallmarks of ALS could further improve our insights on disease onset, pathogenesis and progression and might contribute to the design and evaluation of novel drugs and therapies, by monitoring their effects in vivo.

Radiotracers applied in ALS

Although a small variety of both PET and SPECT tracers has been proposed to visualize disease specific changes [49, 60], the majority of published radionuclide imaging studies in ALS have used [^{18}F]-fluorodeoxyglucose ([^{18}F]-FDG), visualizing cerebral glucose metabolism. Already in 1987, Dalakas et al. showed decreased [^{18}F]-FDG uptake, indicating neuronal malfunctioning in the ALS brain [61]. Later, several groups confirmed hypometabolism in the primary motor cortex, supplementary motor cortex and premotor cortices but also in the frontal and parietal cortex. It was hypothesized that the degree of hypometabolism in the motor cortex correlates with disease duration, but conflicting results have been reported [62-64]. On the other hand, hypermetabolism was observed in the mesiotemporal cortex, cerebellum and upper brain stem [65]. Overall, diagnostic value analysis of [^{18}F]-FDG PET versus a control population showed 90-95% sensitivity [66-68]. In the future, implementation of [^{18}F]-FDG PET in the standard diagnostic work-up will result in large datasets, allowing multivariate analysis and identification of spatially distinct brain networks affected by neurodegeneration. Only very recently, Pagani et al. showed that spatial individual component analysis, which determines significant discriminative regions, in combination with multivariate analysis resulted in 99% accurate differentiation between patients and controls [69]. These results indicate that the degenerative process affects specific brain connections rather than isolated brain regions and are in line with a previously describes hypothesis of anterograde disease propagation along specific tracts [70]. In ALS with co-morbid cognitive impairment or FTD, prefrontal, anterior cingulate and insular hypometabolism was observed when compared with cognitively normal ALS patients [71, 64, 25]. As mentioned before, [^{18}F]-FDG may also be a valuable prognostic tool as a predictor for cognitive impairment or FTD, which decreases survival [72, 73]. A potential drawback of [^{18}F]-FDG is that its uptake might not only represent neuronal activity but the signal is also derived from astrocytes. Furthermore, Pellerin and Magistretti showed that glutamate uptake in astrocytes stimulates glucose utilization, suggesting direct coupling between ongoing excitotoxicity and increased [^{18}F]-FDG uptake [74].

A second indirect target applied to quantify brain dysfunction in ALS is cerebral blood flow (CBF). Using [^{15}O]H_2O PET, [^{99m}Tc]-d,1-HMPO (hexamethyl-propyleneamine oxime) SPECT or [^{99m}Tc]-ECD (ethyl cysteinate diethylester) SPECT, a decrease in CBF has been observed in the primary motor cortex of ALS patients [75, 76], as well as impaired CBF in the frontal lobes of ALS patients with co-morbid cognitive involvement [77]. The extent of cortical changes, as determined by [^{99m}Tc]-ECD SPECT, correlated with the degree of functional disability and the location of motor impairment [78].

To investigate the hypothesis of mutual pathogenic processes in both ALS and Parkinson's Disease, imaging of the dopaminergic nigrostriatal tract, using [^{18}F]-3,4-dihydroxyphenylalanine ([^{18}F]-DOPA) PET and [^{123}I]IPT (*N*-(3-iodopropen-2-yl)-2β-carbomethoxy-3β-(4-chlorophenyl)tropane) SPECT have shown reduced striatal presynaptic dopaminergic functioning in ALS patients when compared to healthy volunteers, suggesting subclinical disturbance of the nigrostriatal tract. However, contrasting results were reported on whether this decrease in tracer uptake correlates with disease duration [79-81].

Flumazenil is an antagonist of the gamma-amino butyric acid, subtype A (GABA-A) receptor that binds its benzodiazepine subunit. Since the receptor is expressed on both pyramidal cells and interneurons of the cerebral cortex, [^{11}C]-Flumazenil is a potential in vivo marker for neuronal loss and potential GABA-ergic inhibitory dysfunction in ALS and therefore, provides important evidence for inhibitory failure with potential consequent excitotoxicity as pathologic hallmark. Several imaging studies using [^{11}C]-Flumazenil have been performed in ALS. In 2000, Lloyd et al. showed significantly reduced tracer distribution volumes in both motor and extra-motor regions of the ALS brain [82]. Furthermore, the pattern of reduced [^{11}C]-Flumazenil distribution volumes in patients with fALS, harboring the D90A mutation in the SOD1 gene, differed from the pattern observed in sALS cases [83], in which a correlation between decreased tracer uptake and poorer verbal fluency was reported [84]. Although ALS and PLS show similar overall [^{11}C]-Flumazenil patterns when compared to healthy controls, PLS patients showed more extensive neurodegeneration in the primary motor cortex and anterior cingulate cortex [85]. Therefore, [^{11}C]-Flumazenil was hypothesized to be useful to discriminate between ALS and PLS.

In 2005, Turner et al. investigated the distribution of [^{11}C]-WAY100635 (*N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]- *N*-(2-pyridyl)cyclohexanecarboxamide), an antagonist for the serotonin 1A (5-HT1A) receptor in ALS patients. [^{11}C]-WAY100635 PET revealed widespread cerebral decrease of tracer uptake in both motor- and extra-motor regions of the ALS brain when compared to healthy controls, with the most prominent decrease in frontotemporal regions [86]. These results suggest potential involvement of the serotonergic system in ALS pathogenesis.

Promising novel targets and tracers

Neuroinflammation

Chronic neuroinflammation is characterized by activated microglia (gliosis) and astrocytes (astrocytosis), and is a common feature in several neurodegenerative diseases. During the past decade, great attention is given to the involvement of this inflammatory response in ALS pathogenesis and progression [87, 44]. As resident macrophages of the CNS, microglia are involved in the innate immune response and turn into their activated state in case of tissue damage, pathogen invasion or protein aggregation. Initially, neuroinflammation is a protective mechanism prohibiting motor neuron death by stimulating tissue repair. In more advanced disease stages however, this protective immune response shifts towards a sustained, neurotoxic inflammatory condition, resulting in a vicious and destructive cycle of motor neuron death, which contributes to rapid disease progression [88]. Imaging of activated microglia may be a valuable diagnostic marker both at onset and during progression of neurodegeneration. It has been shown that neuroinflammation is already ongoing before symptom onset [89, 90], indicating its potential as a marker of the disease in its early stages.

Microglia and astrocytes express various cell surface receptors, cell signaling molecules and mitochondrial membrane receptors, involved in their normal function and regulation. When microglia turn into an activated state, the expression of some of these receptors is upregulated. This constitutes an ideal candidate target for both imaging and potentially also therapy, as neuroinflammation levels are absent or very low in healthy tissue and highly elevated in pathological conditions.

Translocator protein (peripheral benzodiazepine receptor)

The 18 kDa translocator protein (TSPO), previously known as the peripheral benzodiazepine receptor, is located on the outer mitochondrial membrane of microglia, where it is part of the mitochondrial permeability transition pore. Although the exact function is still unknown, TSPO is involved in a variety of cellular processes such as proliferation and apoptosis [91]. Furthermore, TSPO enables cholesterol transport over the mitochondrial membrane and is therefore associated with steroid production [92]. TSPO is hardly expressed in healthy CNS while expression levels are substantially elevated in activated glial cells [93]. At this moment, TSPO is the most extensively explored marker for neuroinflammation PET. However, the Ala147Thr TSPO polymorphism results in three distinct affinity patterns (high affinity binders (HAB), low affinity binders (LAB) and mixed affinity binders (MAB)), showing significant differences in tracer uptake [94, 95]. Therefore, genotyping is required to allow reliable quantification and interpretation of TSPO scans.

The first tracer applied for TSPO imaging was the enantiomeric isoquinoline carboxamide derivative [^{11}C]-(R)-PK11195 [96]. Already in the 1980's, this non-benzodiazepine TSPO ligand was characterized and validated as a promising tracer for neuronal damage [97-100]. Afterwards, [^{11}C]-(R)-PK11195 was

consistently used for imaging of neuroinflammation in disorders such as stroke [101, 102], Alzheimer's disease [103], and MS [104]. In ALS, post-mortem autoradiographic analysis revealed increased [^{3}H]-(R)-PK11195 accumulation in the spinal cord [105] which is in line with previous immunohistochemical analyses [106, 107]. In 2004, Turner et al. [108] reported significantly increased [^{11}C]-(R)-PK11195 uptake in the motor cortex, pons and dorsolateral prefrontal cortex of ALS patients when compared to healthy controls. Furthermore, they found a positive correlation between tracer uptake in the motor cortex and UMN involvement. Despite these promising results on group level, individual tracer uptake was variable with strongly overlapping binding potentials between patients and healthy volunteers, and did not correlate with the degree of motor neuron impairment (ALS-FRS) nor with disease duration. As [^{11}C]-(R)-PK11195 displays high non-specific binding and limited target-to-background ratio, improved potential radiotracers are being tested.

Zürcher et al. successfully applied [^{11}C]-PBR28, a phenoxy-arylacetamide derivative, to quantify neuroinflammation in ALS patients [109]. In this study, increased [^{11}C]-PBR28 uptake was found in the bilateral motor cortices, including the primary and supplementary motor cortices of ALS patients when compared to controls. Standardized Uptake Values (SUV) of the right precentral gyrus were positively correlated with UMN involvement, which is in line with the observations of Turner et al. [108], and negatively correlated with ALS-FRS. Although the sample size was rather small, these findings are promising since abnormal findings were present in all individual subjects with little overlap with controls.

[^{18}F]DPA-714 is a pyrazolo[1,5-a]pyrimidine derivative with improved binding affinity compared to [^{11}C]-(R)-PK11195 [110, 111]. The tracer was tested in various preclinical models of neuro-inflammation, such as mice with cerebral ischemia and chemically induced brain injury, showing significantly increased, TSPO-mediated tracer binding in affected brain regions [110-112]. In healthy humans, brain uptake peaked five minutes post-injection and most of the tracer was localized in the thalamus [113]. In 2012, Corcia and co-workers compared [^{18}F]DPA-714 distribution in ALS patients with tracer distribution in healthy volunteers [114]. They reported that microglial activation is not limited to the motor areas but was also present in the temporal cortex which is at odds with frontal findings in Turner's study [108]. The authors hypothesized that these discrepancies might be explained by differences in location and extent of microglial activation over the course of disease.

P2X7 receptor

A novel target of high interest for neuroinflammation imaging is the P2X7 receptor, which belongs to the purinergic receptor family (P2), consisting of an ionotropic (P2X) and a metabotropic (P2Y)

subfamily. The P2X subfamily consists of low affinity, ATP-gated, non-selective cation channels, selectively expressed on cells originating from the hematopoietic cell lineage, such as microglia, astrocytes and Schwann cells, and is implicated in a variety of pathophysiological CNS functions [115]. P2X7 requires high ATP concentration for its activation and, upon continuous activation, the ion channel changes its conformation towards a membrane pore, permeable to molecules up to 9 kDa without receptor desensitization [116]. When cell injury or tissue damage occurs within the CNS, extracellular ATP levels are rising, leading to P2X7 receptor activation and secretion of pro-inflammatory mediators, such as interleukin-β (IL- β) and tumor necrosis factor- α (TNF-α), from activated microglia, causing receptor shift towards the pore state and induction of a destructive inflammatory cycle, contributing to neurodegeneration [117]. Yiangiou et al. showed upregulated P2X7 expression in microglia of ALS patients using immunohistochemistry [118]. However, experimental evidence in SOD-1 mutated P2X7 KO mice suggests a protective role for P2X7 at some point in ALS pathogenesis [119], indicating a dual effect of the receptor on disease progression depending on disease stage. Next to neuroinflammation, P2X7 receptor signaling has been implicated in excitatory synaptic transmission regulation by modulating release of neurotransmitters, such as glutamate and GABA, from astrocytes [120, 121]. Since P2X7 is related to neuroinflammation and excitotoxicity, both generally accepted key pathogenic mechanisms of neurodegenerative disease, they are considered highly suitable targets for therapeutic exploitation and imaging. Several P2X7 antagonists have been developed, patented and tested over the last two decades [122-124].

Up until now, most published studies evaluating novel P2X7 antagonists have used tritiated compounds to perform mechanistic receptor studies in vitro [125-128]. All tested molecules showed high receptor affinity and, saturable and receptor-mediated binding. Nevertheless, none of these molecules was tested in the clinical setting. An important obstacle towards clinical translation of P2X7 targeting PET tracers is the inter-species difference in receptor binding affinity for rat, mouse and human receptors [129]. Therefore, the use of humanized animal models and human in vivo imaging studies are required. Recently, Ory et al. reported the preclinical evaluation of [^{11}C]-JNJ-54173717 in a humanized rat model and non-human primates [130]. The tracer showed nanomolar affinity for both the rat and human receptor (IC_{50} = 7.6 and 4.2 nM, respectively) with negligible non-specific binding. High receptor-mediated tracer accumulation was observed in the hP2X7 expressing striatum of rats and homogenous specific tracer uptake was observed throughout the brain of non-human primates.

Janssen et al. reported the first preclinical in vivo evaluation of the P2X7 receptor antagonist-based PET tracer [^{11}C]-A-740003 [131]. Despite promising in vitro results, limited brain uptake was observed at all time points. The authors argued that this might be explained by blood brain barrier blocking or

by low receptor expression in healthy brain and additional studies in neuroinflammation models will have to elucidate the tracers potential for neuroinflammation imaging. Given its excellent affinity for hP2X7 (IC_{50} = 3 nM) [132] and proven safety and tolerability in humans [133], [^{11}C]GSK1482160 is an attractive candidate for in vivo visualization of neuroinflammation by P2X7 targeting. Therefore, clinical PET studies should be performed in ALS patients and healthy controls to determine its potential as P2X7 tracer. Next to P2X7, other members of the purinergic receptor family, such as P2X4 and P2Y12 are involved in the pathogenesis of ALS, and therefore constitute further potential targets for in vivo imaging.

Other potential targets and tracers

Cyclo-oxygenase 2 (COX-2) is an inducible enzyme with a key role in the neuroinflammatory cascade, with low levels in healthy tissue and both central and peripheral nervous system upregulation upon inflammation induction [134]. A seven-fold and four-fold upregulations of respectively COX-2 mRNA and protein levels was observed in spinal cord of SOD1 mice and in human spinal cord slices post-mortem [118, 135]. Furthermore, the level of COX-2 upregulation matched with the extent of motor neuron loss and administration of COX-2 inhibitors has been suggested to delay symptom onset in a transgenic mouse model of ALS [136, 137]. These findings clearly highlight the potential value of COX-2 as a target for therapy and disease monitoring. A variety of COX-2 inhibitor based radiotracers, such as [^{11}C]celecoxib and ^{11}C or ^{18}F labeled celecoxib derivatives, have been described [138, 139]. Unfortunately, most of these tracers showed poor in vivo specificity and the search for a suitable COX-2 tracer is still ongoing.

Mono-amine oxidase (MAO)-B is an enzyme, responsible for oxidative deamination of mono-amines such as adrenaline, dopamine and serotonin, and is found mainly in astrocytes and serotonergic neurons. Since neuro-inflammation consists of both microgliosis and astrocytosis, MAO-B represents a potential imaging target. Already in 1995, Fowler et al. showed the possibility to image MAO-B activity using [^{11}C]-deprenyl-D2 ([^{11}C]-DED), a selective and irreversible MAO-B inhibitor [140]. Post-mortem in vitro autoradiography studies on brain slices of ALS patients revealed increased [^{3}H]-DED accumulation in the motor neuron laminae and corticospinal tracts, which correlated with astrocyte count in these brain areas [141]. Furthermore, increased [^{11}C]-DED uptake, associated with astrocytosis, was shown in a complex ALS case [142], confirming the tracers potential as astrocytosis marker. Johansson et al. showed increased tracer uptake in the pons and white matter tracts of ALS patients when compared to healthy volunteers, possibly representing astrocytosis. However, the sample size was rather small and the differences in SUV values between patients and healthy controls were not significantly different [143].

Type 2 cannabinoid receptors (CB2R) belong to the rhodopsin-like family class A of G-protein coupled receptors and are expressed on cells of the immune system. Since CB2R is upregulate in the spinal cord of ALS patients [118] and cannabinoid action has been shown to downregulate NO production by microglia [144], CB2R exhibits potential as a marker and as a therapeutic target for ALS and other neurodegenerative diseases involving neuroinflammation. Several ^{11}C and ^{18}F labeled CB2R tracers have been recently described. [^{11}C]NE40, a 2-oxoquinoline derivative, is shown to exhibit specific and irreversible in vivo binding to human CB2R [145, 146], but failed to show a neuroinflammation signal in AD (Ahmad et al, in press). [^{11}C]KD2, a 4- oxoquinoline derivative displayed receptor mediated binding in ALS spinal cord, as shown by in vitro autoradiography [147]. Slavik et al. recently reported two KD-2 based radiotracers ([^{11}C]RSR-056 and [^{11}C]RS-016) with improved binding affinities towards CB2R and increased uptake in a murine neuroinflammation model [148, 149].

Excitotoxicity

Excitotoxicity by excessive glutamate signaling is considered a key mechanism in neurodegenerative disorders and ,in particular, in ALS pathogenesis. Interestingly, excitotoxicity seems connected to other key pathological mechanisms, such as neuroinflammation and protein aggregation, through increased [Ca^{2+}] influx by activation of specific receptors, such as P2X7, resulting in a variety of cellular processes [150]. Therefore, specific components of the glutamatergic cascade could be of interest to exploit for imaging and potentially drug therapy.

The family of glutamate receptors contains ionotropic ligand-gated ion channels and three groups of metabotropic GTP-gated receptors. The metabotropic glutamate receptor subtype 5 (mGLuR5) is part of the group 1 mGluRs and exhibits a variety of functions within the CNS. They are abundantly expressed on healthy neurons throughout the spinal cord while expression levels on non-reactive glial cells are low. However, these expression levels showed a seven-fold increase in both grey and white matter, as determined by immunohistochemistry, when glial cells shift towards their reactive state [151]. ([^{18}F]3-fluoro-5-(2-pyridylethynyl)benzonitrile) [^{18}F]FPEB PET is a potent and selective mGluR5 inhibitor that generates a stable and long-lived PET signal which is in line with the location of mGluR5 expression, as determined by ex vivo autoradiography [152]. Recent work of Brownell et al. described the in vivo visualization of enhanced mGluR5 expression in the brain and, to a lesser extent, in the spinal cord of SOD-1 mutated mice, using [^{18}F]FPEB PET [153]. Interestingly, [^{18}F]FPEB uptake increased over the course of disease along with the level of neuro-inflammation, as assessed by parallel [^{11}C]PBR28 PET. These results clearly indicate a close connection or even an alliance between

excitotoxicity and neuro-inflammation in ALS pathogenesis, thereby supporting the interplay between the key pathological mechanisms, as described by Sperlágh et al. [150].

The second member of the group 1 mGluR subfamily is the metabotropic glutamate receptor subtype 1 (mGluR1), which is involved in a variety of intracellular signaling systems. As for mGluR5, activation of mGluR1 has been linked to excessive and abnormal glutamate production, suggesting a role in ALS pathogenesis [154]. Downregulation of mGluR1 in SOD-1 mutated, ALS prone mice resulted in later disease onset and progression, and prolonged survival [155]. Furthermore, these mice showed less activated microglia and astrocytes subscribing the link between excitotoxicity and neuroinflammation and the involvement of mGluR1 in ALS pathogenesis. Therefore, also mGluR1 is a possible target for non-invasive visualization of ongoing neurodegeneration in ALS. Unfortunately, the development of suitable mGluR1 PET tracers has been challenging because of cross-reactivity of most developed tracers with mGluR5 [156, 157]. Recently, Zanotti-Fregonara et al. investigated the in vivo use of [^{18}F]-FIMX in the human brain. [^{18}F]-FIMX shows fast brain kinetics and distribution volumes could be estimated [158].

Synaptic density

Altered synaptic density is a pathological feature associated with various neurodegenerative and psychiatric disorders [159-162]. Since excitotoxicity and GABA-ergic inhibitory dysfunction are key players in ALS, disruption of synaptic transmission may be involved in its pathogenesis. Indeed, early synaptic rearrangement, with inhibitory synapse bouton loss and excitatory synapse bouton gain, was observed in motor neurons of SOD-1 mutated mice and favored disease progression [163]. Another study showed that UBQLN2 (a gene linked to ALS and FTD) transgenic mice, developed inclusions associated with synaptic dysfunction and cognitive decline [164]. Up until now, quantification of synaptic density is performed on brain sections obtained from autopsy or resection using antibodies targeting proteins located on synaptic vesicles, such as synaptophysin [165]. However, in vivo quantification of synaptic density could provide important real-time information and might improve early diagnosis. Synaptic vesicle glycoprotein 2A (SV2A) is a transmembrane protein found in all vertebrate synaptic vesicles of both excitatory and inhibitory synapses and is hypothesized to be a key player in exocytosis and neurotransmission control, although its exact role remains unclear [166]. Several SV2A targeting tracers, such as [^{18}F]-UCB-H, [^{11}C]-UCB-A and [^{11}C]-UCB-J have been described [167-169], with [^{11}C]-UCB-J showing the best characteristics for in vivo brain imaging, namely: excellent brain uptake (with the highest uptake values in striatum and cortex), rapid kinetics and metabolism, and suitable time-activity curves [168]. Very recently, Finnema et al. published a first-in-human study in which they confirmed SV2A specific tracer binding and observed that the tracer can indeed visualize

changes in synaptic density in epilepsy patients [170]. Taken together, these observations suggest that SV2A targeting PET tracers harbor great potential for early diagnosis and therapy monitoring of neurodegenerative disorders.

Protein aggregation

Up to now, there are no tracers available for specific protein aggregates in the brain of ALS patients, such as TDP-43, SOD1 and neurofilament deposits [171-174], but several initiatives are being undertaken such as the FTD biomarker initiative from the Association for Frontotemporal Degeneration (http://www.theaftd.org/research/ftd-biomarkers-initiative). Visualization of amyloid-beta deposits in ALS has been investigated concerning a suggested link between ALS and Alzheimer's disease [175]. This relation between ALS and amyloid precursor protein was observed in rodent models and recent post-mortem immunohistochemical analysis have also shown amyloid deposition in 35-50% of all ALS patients, even without co-morbid dementia [176, 175]. Currently, there are 3 published case reports of ALS patients with co-morbid dementia scanned with [^{11}C]-PIB (Pittsburgh Compound B). These reports suggest a potential role for [^{11}C]-PIB PET in the discrimination of various neurodegenerative proteinopathies such as Alzheimer's and ALS with co-morbid frontotemporal dementia (FTD) [142, 177]. Very recently, Matías-Guiu et al. studied amyloid deposition in a cohort of ALS patients using [^{18}F]-florbetaben PET. However, only a small number of patients showed increased tracer uptake in a variety of brain regions and the detected amyloid load in ALS patients was comparable to amyloid load observed in age-matched controls [178]. Therefore, cautious interpretation of these data and further investigations are required.

Mitochondrial dysfunction and oxidative stress

Oxidative stress and mitochondrial dysfunction are both considered key players in ALS pathogenesis [179, 180]. These two processes cannot be considered separate entities. Oxidative stress arises from an imbalance between ROS production and elimination in which the balance is tilted away from elimination and towards excessive production. Damage to mitochondrial respiratory chain proteins can induce an over-reductive state, characterized by electron leakage leading to dramatically increased ROS production. Since excessive ROS accumulation is observed in the CSF of ALS patients, mitochondrial over-reductive state (and its consequent oxidative stress) might be a promising novel target in ALS imaging.

[^{62}Cu]-ATSM ([^{62}Cu]diacetyl-bis(N^4-methylthiosemicarbazone) is a PET radioligand that will be retained in cells displaying redox imbalance, due to copper reduction to Cu(I) [181]. Therefore, it is capable of visualizing cells with mitochondrial dysfunction, such as hypoxic and anoxic cells, seen their

intracellular overreductive state. However, the tracer does not visualize cells harboring sufficient defense mechanisms to counteract ROS production such as prostate cancer cells, which apply the fatty acid synthesis pathway to improve their redox balance. Therefore, inhibition of this pathway results in a significant increase in [^{64}Cu]-ATSM uptake in prostate cancer cells, highlighting the effect of ROS defense mechanisms on tracer uptake in hypoxic cells [182]. In vitro characterization showed increased tracer accumulation in cells in the overreductive state due to mitochondrial dysfunction when compared to normal cells [183, 184] and the feasibility of in vivo oxidative stress detection using this tracer, was shown in patients suffering from mitochondrial disease [185]. In 2011, Ikawa et al. reported increased striatal [^{62}Cu]-ATSM uptake in patients with PD when compared to healthy controls, the tracer showed rapid blood brain barrier penetration and tracer accumulation correlated linearly with disease progression [186]. More recently, the same group showed elevated normalized SUV's in cortical regions, such as the motor cortex bilaterally and the right superior parietal lobe, in 12 ALS patients. Furthermore, increased oxidative stress in these regions was associated with the degree of motor neuron degeneration, represented in the ALS-FRS [187]. Next to its potential for imaging, [^{Nat}Cu]-ATSM has shown potential as a therapeutic agent in mouse models of ALS. Treatment with [^{Nat}Cu]-ATSM effectively protected ALS-prone, SOD1 mutated mice from developing ALS, with an extended survival of 18 months on average. In other studies using different SOD1 mutated mouse models [^{Nat}Cu]-ATSM treatment extended their life with 15% and 26%, respectively [188-190]. Taken together, these observations highlight the promising character of Cu-ATSM in ALS therapy and diagnostics.

Conclusions and future prospects

ALS is a multi-system disorder with a complex underlying pathophysiology rendering adequate diagnosis and management a real challenge. Therefore, implementation of non-invasive in vivo imaging in the diagnostic regimen might improve accuracy of early diagnosis. Currently, [^{18}F]-FDG PET is a promising indirect measure of neuronal dysfunction in ALS that might allow identification of specifically affected connections between brain regions and spatially distinct networks with significant discriminative values. Since neurodegeneration is a process of networks rather than isolated regions, brain network analysis might significantly improve accurate diagnosis. Next to determination of the spatial metabolic signature, in vivo visualization of the multiple underlying pathological processes is likely to aid in gaining directly relevant information on disease onset or progression. In the past decade, several novel radiotracers have been described to visualize, among others, neuroinflammation and excitotoxicity (table 1). For other central molecular processes, involved in ALS pathogenesis such as protein deposition (e.g. TDP-43), tracer development in currently ongoing. In families harboring

specific ALS-causing mutations, imaging of the pathological process initiated by this mutation could allow early disease detection and even prevention when performed longitudinally. Combined application of tracers, targeting different pathological processes, might provide novel insights in disease patterns specific for ALS, PLS or other mimic syndromes, thereby facilitating their differentiation. Finally, clinical implementation of these tracers will allow non-invasive evaluation of novel drugs, counteracting specific pathological pathways, and improve detailed disease progression monitoring. Although not discussed in depth in this manuscript, more widespread implementation of simultaneous hybrid imaging systems, such as PET-MR scanners, might also aid in developing multi-parametric markers and in functional connectivity assessment for further characterization of affected brain networks.

Compliance with Ethical Standards

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Table 1: Overview of tracer molecules applied for ALS imaging

Pathological hallmark	Target	Tracer	References
Glucose metabolism	GLUT (predominant GLUT 1)	[^{18}F]-FDG	61-69, 74
Cerebral blood flow		[^{15}O]-H_2O	75-78
		[^{99m}Tc]-d,1-HMPO	
		[^{99m}Tc]-ECD	
Dopaminergic nigrostriatal tract	Dopaminergic cells	[^{18}F]-DOPA	79
		[^{123}I]-IPT	80-81
Serotonergic system	5-HT1A Receptor	[^{11}C]-WAY100635	86
Neuro-inflammation	TSPO	[^{11}C]-(R)-PK11195	94-108
		[^{11}C]-PBR28	109, 150
		[^{18}F]-DPA-714	110-114
	P2X7 Receptor	[^{3}H]-A-804598	125
		[(3)H]-JNJ-54232334	126
		Compound-17	127
		Compound-13	128
		[^{11}C]-JNJ-54173717	129
		[^{11}C]-A-740003	131
		[^{11}C]-GSK1482160	132-133
	COX-2	[^{11}C]-celecoxib	138
		[^{11}C]/ [^{18}F]-celecoxib derivatives	139
	MAO-B	[^{11}C]-deprenyl-D2	140-143
	CB2R	[^{11}C]-NE40	145, 146
		[^{11}C]-KD2	147
		[^{11}C]-RS-016	148
		[^{11}C]-RSR-056	149
Excitotoxity	mGluR5	[^{18}F]-FPEB PET	150-153
	mGluR1	[^{18}F]-FIMX	158
Synaptic density	SV2A	[^{11}C]-UCB-A	167
		[^{18}F]-UCB-H	169
		[^{11}C]-UCB-J	168-170
Inhibitory dysfunction	GABA A-R	[^{11}C]-Flumazenil	82-85
Protein aggregation	Beta-amyloid	[^{11}C]-PIB	142, 177
Mitochondrial dysfunction	Mitochondria	[^{62}Cu]-ATSM	181-190