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Comprehensive assessment of dendritic spine dynamics in the primary motor cortex of the SOD1^{G93A} transgenic mouse model of ALS

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Table of Contents

Zusammenfassung

Die Amyotrophe Lateralsklerose (ALS) ist eine fortschreitende und verheerende Motoneuron-Erkrankung mit einer durchschnittlichen Überlebenszeit von 3-5 Jahren vom Auftreten der Symptome bis zur invasiven Atemunterstützung oder zum Tod. Klinisch ist sie durch das gleichzeitige Vorhandensein von Zeichen der oberen und unteren Motoneuronschädigung bei der neurologischen Untersuchung gekennzeichnet, beispielsweise einer Hyperreflexie in Kombination mit Muskelschwäche und Faszikulationen.

Obwohl die Degeneration der oberen Motoneurone (engl. upper motor neurons, UMNs) in den letzten Jahrzehnten in Gehirnproben von ALS-Patienten nachgewiesen wurde und als charakteristische Manifestation der ALS-Krankheit gilt, scheint die Degeneration der UMNs in Mausmodellen der Krankheit weniger auffällig zu sein, insbesondere im präsymptomatischen Stadium. *In vitro* Untersuchungen haben jedoch strukturelle Defekte von sogenannten dendritischer ‚Dornen', der UMN ergeben, was zu der Annahme führte, dass ALS eine Synaptopathie darstellt. Dendritische Dornen sind die strukturellen Korrelate von Postsynapsen und der primäre Orte des Erregungseingangs einer Nervenzelle. Über die dynamischen Veränderungen der Dornen von UMNs und die Auswirkungen von Fortbewegung und motorischem Lernen auf diese ist jedoch wenig bekannt. Daher habe ich hier die Auswirkungen neurodegenerativer Prozesse auf die apikalen dendritischen Dornen von Schicht-V-Pyramidalneuronen (engl. layer V pyramidal neurons, LVPNs) im primären motorischen Kortex unter Ausgangsbedingungen und während eines Paradigmas zum Erlernen motorischer Fähigkeiten in 5-6 Monate alten präsymptomatischen $hSOD^{G93Adl}$-transgenen Mäusen (SOD) untersucht. Die Bilder wurden chronisch mit einem Zwei-Photonen-Mikroskop an wachen, sich verhaltenden Mäusen aufgenommen. Unter Ausgangsbedingungen zeigten die Ergebnisse eine Abnahme der Dornendichte bei SOD-Mäusen im Vergleich zur Kontrollgruppe (CTR), und auch die Dornendynamik, einschließlich der Bildungs-, Eliminations- und Umsatzrate, war in der SOD-Gruppe im Vergleich zur CTR-Gruppe geringer. Darüber hinaus hatten die

Mäuse der SOD-Gruppe nach 4 Tagen eine höhere Überlebensrate, was darauf hindeutet, dass die Stacheln stabiler waren.

Verhaltentsuntersuchungen im ‚offenen Feld' zeigten ein vermindertes Erkundungsverhalten und eine geringere Fortbewegung bei präsymptomatischen SOD-Mäusen, was sich in einer signifikant geringeren Gesamtdistanz, einer geringeren Durchschnittsgeschwindigkeit und einer geringeren Anzahl von Eintritten in den zentralen Bereich der Arena zeigte. Das verringerte Erkundungsverhalten war nicht auf tatsächliche motorische Funktionsdefizite zurückzuführen, da der SOD-Score dieser präsymptomatischen Mäuse Null war. Außerdem zeigte die anschließende Laufleistung in einem Standard- und einem komplexen Rad keinen Unterschied zwischen den beiden Gruppen, was ebenfalls das Fehlen motorischer Defizite in der SOD-Gruppe im präsymptomatischen Stadium bestätigt. Stattdessen ist es wahrscheinlicher, dass sie auf Beeinträchtigungen in anderen Hirnregionen als dem motorischen Kortex zurückzuführen sind.

Anschließend untersuchte ich die strukturelle Plastizität während eines 50-minütigen Kurzzeitintervalls während die Mäuse auf einem Styroporball liefen. Die Ergebnisse zeigen, dass die Umsatzrate der Dornen in der SOD-Gruppe während des Kurzzeitintervalls signifikant niedriger war als bei den Kontrolltieren. Außerdem waren die dynamischen Veränderungen der Dornen umso größer, je länger die Mäuse auf dem Styroporball liefen. Diese Korrelation wurde jedoch nur in der CTR-Gruppe, nicht aber in der SOD-Gruppe beobachtet, was auf einen Mangel an schnellen Veränderungen der Dornen bei SOD-Mäusen hinweisen könnte.

Die Dornendynamik wurde dann bei einer freiwilligen motorischen Lernaufgabe untersucht, bei der es sich um ein speziell entwickeltes Standardrad, welches durch spätere Manipulation zu einem ‚komplexen' Rad umgewandelt wurde. CTR-Mäuse zeigten eine erhöhte Rate der Dornenbildung nach 2 Tagen motorischen Lernens, jedoch nicht in der SOD-Gruppe, was ebenfalls ein Defizit in der Dornenbildung aufweist.

Insgesamt ist dies die erste umfassende *in vivo* Studie kortikaler UMN Synapsen in einem ALS Mausmodell. Mit meinen Versuchen konnte ich eine Abnahme der Dichte

als auch eine starke Einschränkung der strukturellen Plastizität synaptischer Dornen von UMNs in dem transgenen hSOD^{G93Adl}-Mausmodell im präsymptomatischen Stadium zeigen. Meine Ergebnisse untermauern somit das Konzept der ALS als Synaptopathie.

Summary

Amyotrophic lateral sclerosis (ALS) is a progressive and devastating motor neuron disorder with an average survival time of 3-5 years from the onset of symptoms to invasive respiratory support or death[1,2]. It is clinically characterized by the co-existence of upper and lower motor neuron signs on neurological examination, for example, hyperreflexia combined with muscle weakness and fasciculations[3,4].

Although the degeneration of upper motor neurons (UMNs) has been detected in brain samples from ALS patients over the past decades and is considered to be a characteristic manifestation of the disease, degeneration of UMNs appears to be less prominent in mouse models of the disorder, especially at the presymptomatic stage. However, evidence from *in vitro* studies has now revealed structural defects of dendritic spines of UMNs, which led to the notion that ALS might be a synaptopathy[5]. Dendritic spines are the structural correlates of postsynapses and are the primary sites of excitatory input [6]. However, little is known about the dynamic changes of spines of UMNs under baseline conditions and the effects of locomotion and motor learning on their dynamics. Therefore, I herein explored the impact of neurodegenerative processes on the apical dendritic spines of layer V pyramidal neurons (LVPNs) in the primary motor cortex under baseline conditions and during motor skill learning paradigm in 5-6-month-old pre-symptomatic $hSOD^{G93Adl}$ transgenic mice (SOD). Images were obtained chronically with a two-photon microscope in awake, behaving mice.

Under baseline conditions, the results showed reduced spine density in SOD mice in comparison to the control (CTR) group, and spine dynamics, including formation, elimination, and turnover rate, were also decreased in SOD mice in comparison to the CTR group. In addition, mice in the SOD group had a higher survival fraction after 4 days, indicating that spines were more stable.

Behavioral testing performed in the open field showed decreased exploratory behavior and locomotion in SOD mice as manifested by a significantly reduced total distance traveled, lower average velocity, fewer entries into the central area of the arena, and an increase in total freezing time. The decreased exploratory behavior was not due to

actual motor functional deficits, as the SOD score of these presymptomatic mice was zero, besides, the subsequent running performance on the standard and complex wheel showed no difference between the two groups, also confirming the absence of motor deficits in the SOD group at the presymptomatic stage. It is instead more likely due to impairments in brain areas other than the motor cortex.

I then further explored the structural plasticity over a 50-minute short-term interval in mice running on a styrofoam ball. Results showed that the spine turnover rate of the SOD group was significantly lower during the short-term interval. Furthermore, the longer the distance the mice ran on the styrofoam ball, the greater the dynamic changes in their spines, however, this correlation was only observed in the CTR group but not in the SOD group, indicating a lack of spine plasticity in SOD mice.

Spine dynamics were then investigated upon exposure to a voluntary motor skill learning task, which is based on a custom-designed standard wheel with a subsequent transition into a complex running wheel. CTR mice showed an increased rate of spine formation after 2 days of motor learning, however, not in the SOD group, demonstrating a deficit in spine formation in SOD mice.

Overall, this is the first comprehensive *in vivo* study on structural dynamics of spines in the motor cortex of an ALS mouse model. I demonstrate a significant reduction both in spine density and dynamics across different modalities of UMNs in the hSOD^{G93Adl} transgenic mouse model at the presymptomatic stage. My data thus provide further insight into synaptic dysfunction of cortical neurons and corroborate the concept of ALS as a synaptopathy.

1. Introduction

1.1 Amyotrophic lateral sclerosis

1.1.1 Clinical manifestations and epidemiology of ALS

Amyotrophic lateral sclerosis (ALS) is the most common, adult-onset motor neuron disease, which is characterized by the degeneration of upper (UMN) and lower motor neurons (LMN)[4,7,8]. In ALS, the site of onset, the progression pattern and rate, and the degree of involvement of UMNs and LMNs are markedly heterogeneous[9-14]. Symptoms most frequently begin focally in the distal limbs, and then the muscle weakness gradually spreads to the proximal limbs, bulbar, and finally, respiratory muscles as the disease progresses. Typically, patients with ALS eventually die of respiratory failure[9-14]. Symptoms onset in ALS is usually asymmetric. Approximately 60-80% of ALS cases present initially with spinal symptoms, such as muscle weakness of the limbs or reduced or absent tendon reflexes; and to a lesser extent, bulbar symptoms are the first to be reported, characterized by dysphagia and dysarthria. Alongside bulbar onset and spinal onset, there are some uncommon forms of onset, such as thoracic onset, respiratory symptoms, dementia, or cognitive changes[15-18]. Extraocular muscles are typically intact in ALS patients, as well as sensory examinations are relatively normal[19-23].

There is at present no effective treatment for ALS. Riluzole and edaravone are the only two drugs approved by the U.S. Food and Drug Administration (FDA). However, these two drugs only have a limited effect in slowing down ALS disease progression[24-26]. Therefore, the main clinical management options for ALS patients are symptomatic and palliative care[27].

Population-based studies report that the incidence of ALS is approximately 2 to 3 per 100,000 people per year[22,28-30]. The prevalence in different regions of the world is approximately 5 per 100,000 people[17,31-34]. Males are more susceptible than females, with an approximate male to female ratio of 1.5:1[1,15,16,35-37]. The average age of onset

of the disease is 55 to 60 years[2,9,12-14,38]. The average age at diagnosis of ALS is around 60 years, due to the atypical initial symptoms and the lack of accurate and efficient diagnostic biomarkers, resulting in a diagnostic delay of about 9-15 months from disease onset[11,18].

1.1.2 Etiology of ALS

1.1.2.1 The multifaceted etiology of ALS

ALS has been recognized as a degenerative neurological disease with multiple causes, including a combination of genetic predispositions and various environmental risk factors[39-42]. The mechanisms mediated by different genetic mutations may not be mutually exclusive and might all converge on common downstream events, including abnormalities in protein homeostasis, glutamate-mediated excitotoxicity, oxidative stress, mitochondrial dysfunction, altered axonal transport, endoplasmic reticulum (ER) stress, disturbed cytoskeletal dynamics, defects of the inhibitory cortical circuits, as well as neuroinflammation. These pathological changes interact with each other and ultimately cause motor neuron degeneration and death in a hitherto incompletely understood manner[39,43-45].

Lately, ALS has been suggested to be characterized by a prolonged preclinical period for years or even decades[46]. Some pathological changes, such as the hyperexcitability of LMNs and the reduction of their dendritic arborization, have been detected even during the embryonic period in ALS rodent models[47]. Furthermore, a population-based study indicates that the onset of ALS involves six different aspects, and proposed a multistep model for ALS[48], in which inherited genetic susceptibility together with other risk factors, including environmental exposure to e.g. heavy metals, pesticides, and chemicals, physical exercise, and age have been implicated in causing long-term neurodegenerative processes, adding complexity to the pathological mechanisms of ALS[39,49].

1.1.2.2 Genetic causes of autosomal dominant ALS

The majority of ALS cases are sporadic (sALS), while approximately 10% of ALS patients have inherited the disease, based on autosomal dominant gene mutations (with a few exceptions that occur recessively), therefore, classified as familial cases (fALS). The fALS and sALS patients are clinically indistinguishable from each other[50]. Owing to the rapid development of genetic analysis techniques, numerous causal genetic mutations are identified in ALS pathogenesis and our knowledge of the genetic basis of ALS is growing steadily. More than 25 genes as of today to cause or increase susceptibility to developing ALS have been identified, such as the gene encoding the Cu/Zn superoxide dismutase 1 (*SOD1*), fused in sarcoma (*FUS*), a GGGGCC (G_4C_2) hexanucleotide repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72*) gene, the transactive response DNA-binding protein (*TARDBP*, encoding TAR DNA binding protein 43 (TDP-43)), and other less common genes [43,51] (Table 1).

Table 1. Genetic mutations in ALS.

Gene mutations	Familial	Sporadic
C9orf72	45-50%	9%
SOD1	12%	1%
FUS	4%	1%
TARDBP	5%	2%
Others & Unknown	40%*	90%*

*[52]

SOD1 was the first causative gene identified in fALS, accounting for ~12% of fALS cases and ~1% of sALS cases[52-54]. *SOD1* is an enzyme that catalyzes the conversion of superoxide radicals into oxygen and hydrogen peroxide. The pathologic processes and mechanisms caused by *SOD1* mutations are still incompletely understood[55]. The detrimental impacts of mutated *SOD1* on neurons are attributed to a toxic gain-of-function (GOF) mediated by intracellular aggregates, causing the sequestration or scavenging of vital cellular components, while a loss-of-function (LOF) of the mutated *SOD1* is less likely involved[54-59].

C9orf72 is the most frequent genetic mutation so far, accounting for ~45-50% of familial cases and ~9% of sporadic cases[52,60-64]. The mutant *C9orf72* gene, located on chromosome 9p21, carries a GGGGCC hexanucleotide repeat expansion in a non-coding intron[63,64]. The pathological processes triggered by *C9orf72* repeat expansions remain incompletely understood. A couple of hypotheses were put forward, such as the haploinsufficiency of *C9orf72* activity, or toxicity conveyed by aberrant RNA foci or through toxic dipeptide repeat proteins (DPRs)[4,63-71].

Mutations within the *TARDBP* gene, which encodes the TDP-43 protein, have been identified in ~5% of familial cases and ~2% of sporadic cases[52,72-75]. TDP-43 is a ubiquitously expressed, heterogeneous nuclear ribonucleoprotein (hnRNP), regulating various cellular processes, including transport of RNA, mRNA splicing, as well as biogenesis of microRNAs[73,74,76-81]. TDP-43 positive inclusions were identified in neuronal cytoplasm in most cases of ALS and FTD independent from genetic mutations, except for cases with mutations in *FUS* or *SOD1*, which have FUS or SOD1positive inclusions, respectively[73,74].

FUS mutations have been found in less than 1% of sALS and approximately 4% of fALS cases[52,82-84]. FUS is also an RNA/DNA binding protein that shares substantial structural and functional features in common with TDP-43[85]. FUS also localizes primarily in the nucleus where it plays a role in regulating RNA metabolism, including mRNA transport, mRNA stability, and RNA splicing[83,86-95]. Toxic GOF caused by the aggregation of FUS and TDP-43, and/or a LOF due to the mislocalization of FUS/TDP-43 from the nucleus to the cytoplasm, preventing TDP-43/FUS from performing its normal nuclear functions[96], have been proposed as pathological mechanisms that lead to motor neuron dysfunction and progressive neurodegeneration in ALS[83,85,97-104].

Some of the other less common disease-causing genes are *optineurin* (OPTN) (also known as 14.7-kDa interacting protein (FIP-2))[105], *granulin* (GRN)[106], *ubiquilin 2* (UBQLN2)[107], *matrin 3* (MATR3), *valosin-containing protein* (VCP)[108], and coiledcoil-helix-coiledcoil-helix domain-containing 10 (CHCHD10)[109], etc.

Although ALS can be caused by mutations in various genes, the molecular pathways mediated by these mutations are not isolated from each other, but instead, share many

common pathways and ultimately lead to neurodegeneration[55]. For example, mutations in genes of some RNA binding proteins, such as *TARDBP* and *FUS*, can lead to misregulation in the RNA metabolism, such as the failure of mRNA splicing[74].

Gene therapy for ALS is evolving as genetic approaches continue to develop and as studies on the genetic aspects of ALS continue to progress. An important breakthrough in recent years has been the development and clinical testing of antisense oligonucleotides (ASOs)[110-117]. As has been evidenced by Polymenidou et al., who utilized a stereotactic injection of ASOs into adult mouse striatum to degrade TDP-43 mRNA *in vivo*, thereby contributing to reducing mutant TDP-43 expression levels.[118].

1.2 Pathophysiology of ALS

1.2.1 Cell-autonomous mechanisms leading to motor neuron degeneration

Decades of research have unraveled numerous intracellular molecular mechanisms which are linked to the degeneration of motor neurons in ALS.

Mutations in genes, such as *SOD1*, *VCP*, and *UBQLN2*, cause protein degradation aberrations, either through impairment of proteasomal pathways or compromised autophagy, or both[74]. Basso et al. found that mutant SOD1 protein could not be completely ubiquitylated, thus degradation by the proteasomal machinery failed[119]. Zhang and co-workers found that the use of trehalose to induce mTOR-independent autophagic pathway decreased SOD1 accumulation, reduced muscle denervation, and delayed disease onset[120]. However, in another experiment, the authors found that activation of the mTOR (mammalian target of rapamycin)-dependent autophagic pathway with rapamycin not only failed to remove abnormal SOD aggregates but also accelerated motor neuron degeneration and disease progression[121], indicating that autophagy in ALS is altered and might represent one of the potential targets for ALS therapeutic strategies.

Misfolded proteins and their toxic aggregates accumulate in the ER lumen, activating

ER stress sensors, which in turn inhibit certain protein synthesis and trigger an "unfolded protein response" (UPR) in an affected cell. Under physiological conditions, this cellular stress pathway is effective in maintaining cellular homeostasis, whereas, in some neurodegenerative diseases, such as ALS, the prolonged ER stress renders the ER incapable of sustaining a homeostatic situation and causes neuronal dysfunction and cell death[122]. Furthermore, Saxena and co-workers found that different subtypes of motor neurons display differential sensitivity or vulnerability to ER stress, as evidenced by the fact that upregulated ER stress markers are first detected in the fast fatigable (FF) motor neurons, which are the most vulnerable spinal motor neurons in the hSOD1^{G93A} transgenic mice[123].

Dysfunction of mitochondria is also among the early cellular pathological events in ALS[124]. Mitochondria can uptake the excessive calcium ions from the cytoplasm and thus relieve Ca^{2+} load and provide adenosine triphosphate (ATP) for important biological processes, such as re-establishing the ion gradients[125]. Ferri et al. found that mutant SOD1 could bind to the mitochondria in motor neurons, leading to increased production of toxic oxyradicals, and triggering cell apoptosis[126]. Moreover, impairment of axonal cytoskeleton and axonal transportation have also been found in ALS. These pathological alterations can cause the denervation of muscles by LMNs, and lead to clinical symptoms of paralysis[127,128].

1.2.2 Circuit mechanisms of ALS

As characterized by a dual degeneration and dysfunction of UMNs and LMNs, the origin of ALS has been a long-standing debate and three theories have been proposed[129,130]. The "dying back" hypothesis, arguing for a neuromuscular junction (NMJ) origin as well as a retrograde progression along the corticospinomuscular axis. Based on this hypothesis, the primary motor cortex (M1) is the final degeneration site and may have a limited contribution to the disease[131,132]; The "independent" hypothesis: ALS was postulated to be an independent and simultaneous degeneration of the UMNs and the LMNs, and spread regionally[44,133]; The "dying-forward" hypothesis proposes

that degeneration originates in M1 and propagates to the downstream targets in a corticofugal manner[134].

Interestingly, hyperexcitability of motor neurons seems to be one of the earliest pathological changes, including UMNs[135-137] and LMNs[138,139], both in transgenic mice[135-139] as well as in ALS patients[140-143]. Glutamate is the primary excitatory neurotransmitter in the central nervous system. It is stored in presynaptic vesicles and released upon depolarization of the membrane[127]. Excitotoxicity can occur when excessive glutamatergic stimulation is occurring, due to either increased release from the presynaptic compartment, or increased postsynaptic stimulation mediated by altered expression of the postsynaptic glutamate receptors (N-Methyl-D-aspartic acid receptor (NMDA receptors)), or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA receptors)) on the vulnerable neurons[127], or compromised glutamate uptake owing to reduced excitatory amino acid transporters (EAAT2) (rodent, glutamate transporter 1 (GLT1)) on astrocytes, which causes glutamate clearance defects. These primary events are believed to cause intracellular Ca^{2+} burden and compromise Ca^{2+}-dependent pathways[124,144], which in turn progressively disrupt normal cellular functions, manifested by dysfunction of proteasome-mediated protein degradation, inducing consecutive ER stress, and mitochondria dysfunction[145]. However, recent *in vivo* patch-clamp experiments in rodent models strongly question the causal involvement of hyperexcitability of LMNs and rather suggested that hypoexcitability was the principal driver in LMNs degeneration[146,147]. Intriguingly, hyperexcitability of the entire motor cortical area is evident in human ALS patients[142,148-150]. It is assessed by means of transcranial magnetic stimulation (TMS) in combination with electromyography (EMG) recordings as a means of measuring the threshold needed to cause a motor potential[142,148-150]. Importantly, these studies also suggest an involvement of cell types other than UMNs in the development of cortical hyperexcitability [129]. To further identify the circuit elements involved in cortical hyperexcitability, let me first delineate the composition of the microcircuitry of M1, which is the area where UMNs reside.

1.2.2.1 Primary motor cortex

The primary motor cortex has been assigned an imperative role in initiating voluntary movements, as well as in acquiring motor skills[151]. In addition, alterations in M1 are very early events in ALS disease progression, however, its contribution has long been underestimated, mainly due to the difficulties in identifying UMNs deficits explicitly[152]. M1 is composed of six layers, although a typical layer four as seen in sensory areas is absent. M1 contains two main types of projection neurons: intratelencephalic neurons (IT-type), which are subdivided into layer 2/3, 5A, 5B, and 6; and pyramidal tract-type neurons (PT-type), which are located in layer 5B[153]. IT-type neurons target other IT-type and PT-type neurons, while PT-type neurons only communicated with other PT-type neurons and don't project to IT-type neurons[154]. The axons of PT-type neurons form the corticobulbar/corticospinal tract, impinging on interneurons in the brainstem and spinal cord in rodents, which then transmit signals to the LMNs and control muscle movements[154,155]. The main input to PT-type neurons is provided by layer 2/3 pyramidal cells.

Neuronal activity in M1 is tightly regulated by a number of reciprocally connected types of inhibitory interneurons. Three major groups of interneurons are known in M1[156]. The most abundant type is parvalbumin (PV)-expressing fast spiking interneurons, which target the somatic compartment of pyramidal neurons[156,157]. Somatostatin (SST)-expressing interneurons mainly target dendrites of pyramidal neurons, it also inhibits PV interneurons[158]. Interneurons expressing the ionotropic serotonin receptor 5HT3a (5HT3aR) are characteristic of the third class of interneurons. The biggest subclass here are interneurons expressing vasoactive intestinal peptide (VIP). VIP interneurons mainly serve a disinhibitory role by inhibiting SSTs and PVs, with a small direct inhibitory input onto pyramidal cells[158].

In addition, cortical motor neurons in M1 are embedded within long-range circuits[154,155]. The two main long-range loops involved in forebrain function are the cortico-basal ganglia-thalamocortical loops and the cortico-cerebellum-thalamocortical loops[154,155].

1.2.2.2 Deficits of M1 circuit elements in ALS

Recently, several landmark studies have helped to elucidate the involvement of the cerebral cortex and the UMNs in the early-stage of ALS pathophysiology. With the help of retrograde labeling, Özdinler and co-workers first demonstrated that loss of cortical spinal motor neurons (CSMNs, also known as UMNs) appears at P30 in a $SOD1^{G93A}$ transgenic mouse model, significantly before the appearance of clinical signs, they further confirmed that this CSMNs loss was not due to defects in axonal retrograde transport[159]. In another mouse model, $SOD1^{G86R}$, loss of CSMNs was also detected earlier than LMNs degeneration and NMJ degeneration[160]. In addition, they revealed a somatotopic relationship between the cortical and spinal lesions, suggesting that early cortical damage may boost LMNs dysfunction and degeneration[160]. Further experiments revealed that inhibition of CSMNs differentiation in $SOD1^{G86R}$ mice by genetic manipulation was found to prevent the development of disease and prolong the lifespan of mice[161].

In addition, an impairment of the intracortical inhibition provided by interneurons is also thought to be implicated in ALS pathogenesis[162]. In ALS patients, a battery of studies conducted by Vucic and co-workers suggested that a reduction in short-interval intracortical inhibition (SICI) could be detected at the presymptomatic stage and could be used to distinguish ALS from mimic disorders[142,163]. A study by Foerster et al. reported lower gamma-aminobutyric acid (GABA) levels in M1 of ALS patients, which indicated a reduction in inhibitory neurotransmission[164]. Notably, alterations of the number of interneurons per se or changes in their electrophysiological properties associated with ALS are not consistent across studies. Nihei et al. first found that PV-interneurons[165] were reduced in M1 of ALS patients[166]. Minciacchi et al., on the contrary, found an increase in PV-interneurons in M1 of $SOD1^{G93A}$ transgenic mice[167]. Moreover, PV-interneurons were hypoactive in the $SOD1^{G93A}$ transgenic mice[168], and in the $TDP\text{-}43^{A315T}$ transgenic mice[169]. The latter group also found that PV hypoactivity was likely a result of increased inhibition from hyperactive Sst-positive interneurons, which in turn is causing a disinhibition of layer V pyramidal neurons (LVPNs).

Furthermore, Özdinler et al. failed to identify any difference in the cortical interneuron populations between the SOD1^{G93A} mice and the littermate controls[159]. Kim et al., however, found increased intrinsic excitability of PV-positive interneurons in SOD1^{G93A} transgenic mice[135], whereas Fogarty et al. studied a combination of miniature synaptic currents and synaptic currents caused by presynaptic release, yet failed to detect any difference of the spontaneous inhibitory postsynaptic currents (sIPSCs) of UMNs[170,171]. Collectively, it seems that the properties of intracortical interneurons and their relevance for UMNs function in ALS require further investigation.

1.2.3 Non-motor system involvement in ALS

The involvement of the non-motor system in ALS is garnering increasing attention, although its full extent remains to be determined.

Approximately 50% of people with ALS exhibit symptoms reminiscent of frontotemporal dementia (FTD), characterized by personality changes, irritability, and frontal lobe executive function deficits[172-176]. ALS and FTD are now generally accepted to belong to the same spectrum[74,177], especially in view of their common genetic causes, such as mutations in the *C9orf72* gene[178]. Furthermore, in terms of pathology, cytoplasmic inclusions composed of the TDP-43[73,179,180], which is proposed to represent a common pathological substrate that connects these neurodegenerative diseases[73], have been identified in glia and neurons in both motor cortex of ALS patients and the frontal and temporal lobes of FTD patients[63,180,181].

Hippocampal involvement in patients with ALS is also becoming widely recognized[182-185]. The major hippocampal pathologies in ALS include cytoplasmic inclusions in the dentate gyrus (DG) and layer II/III of the transentorhinal cortex (TEC), as well as subsequent neuronal loss and gliosis within these cortical regions. These pathological features are specific to ALS and are different from Alzheimer's disease (AD), which primarily involves the entorhinal cortex[185].

Notably, as opposed to its cardinal involvement in human ALS, the degeneration of UMNs seems much less prominent in mouse models of the disease, especially during

the presymptomatic stage. However, in spite of this absence of overt UMNs degeneration, there is now evidence for alterations of electrophysiologic properties and structural deficits of UMNs, which has led to the notion of ALS being a synaptopathy[5]. But little is known about the nature of this synaptopathology on UMNs and its consequences on synapse dynamics under baseline and experience-dependent conditions.

1.3 Synaptic dysfunction in ALS

1.3.1 Synapse and neural signal transmission

Synapses are the communication sites between neurons. There is ample evidence that synapses are affected in numerous neurodegenerative diseases already from early stages [186,187]. I thus here address the impact of neurodegenerative processes on synaptic structures in ALS.

A mature synapse is composed of two compartments: presynaptic and postsynaptic. It contains a presynaptic active zone with synaptic vesicles, a synaptic cleft, and a postsynaptic matrix of proteins composing the postsynaptic density (PSD)[188,189]. The PSD contains synaptic receptors, cytoskeletal components, cell adhesion molecules (CAM), and signaling molecules[190-193]. Out of these proteins, the postsynaptic density protein-95 (PSD-95) is the most abundant scaffold protein[193,194]. The PSD surface area is proportional to the inserted AMPA receptors, thereby indicating synaptic strength[188,195,196]. Neurotransmitters are released from presynaptic vesicles into the synaptic cleft, where they are diffused and bind to the corresponding postsynaptic receptors, thereby facilitating information transmission between the connected neurons[197,198]. The complex geometric structure of the dendritic tree and its active and passive membrane properties enable a neuron to integrate input signals and then relay to the axon initial segment (AIS), where the action potentials (APs) are generated[199]. The basal dendrites and the proximal apical dendrites mainly receive inputs from local circuits, while the apical tufts primarily receive the thalamocortical inputs and

corticocortical inputs. The dendritic spine is the locus where excitatory synaptic inputs are primarily received[200,201].

1.3.2 Dendritic spines

Dendritic spines are the structural correlates of postsynapses and are the main sites of excitatory input[202,203]. These small protrusions from the parent dendrite contact nearby axonal terminals [204]. Because of their unique morphological features with a narrow neck, they provide a spatially isolated compartment[205]. Synaptic strength correlates well with the spine head volume[203,205-208]. Spine head volume varies extremely, ranging from 0.01 to 1μm^3 [202,209,210]. According to their morphology, spines can be divided into four types, where a "stubby spine" is characterized by the lack of a distinct spine neck; a "thin spine" lack a classical large bulbous head, and a "mushroom spine" is distinguished by a large bulbous head and a relatively narrow neck. A filopodia-like protrusion is a long, thin, hair-like structure, often considered to be an immature form of a spine[205,211-213].

The stability of a spine is closely related to its morphology[202,214,215], the spines that survive longer, assumed to be more stable, tend to have a larger spine head size [215-217], while thin spines are more mobile and more plastic[218], and correspondingly, they are less stable[216] and have a shorter survival time[6,214,215]. The lifespan of spines has been demonstrated to vary a lot[216]. They can appear and disappear within tens of minutes during development[219], or they can survive on a time scale of days to months[220,221]. In addition, even stable spines are also volatile structures[222]. Their morphological changes can occur on time scales of seconds to minutes[219,223-225].

Spines undergo structural plasticity to adapt to an ever-changing environment[188,216,226-228]. During the early postnatal development period, spines are highly motile[216]. In the mature adult cortex, spines become relatively stable[229] but remain the ability to strengthen or weaken through enlargement and shrinkage of their size. In addition, they also have the potential to meet functional demands through *de novo* growth and retraction[215,216,218,228]. Therefore, existing synaptic connections in the brain can be modified and new connections between neurons can be established, forming the basis

of the brain to acquire, process and transmit information[203,214,230-233].

It is a general consensus that the formation of spines precedes the emergence of functional synapses, but how quickly they become functional varies from study to study[214]. Kwon et al. found in acute brain slices from young mice (P8-12) that a new spine was induced within 2 minutes upon glutamate uncaging, and functional synapses have been confirmed on them[234]. However, Knott et al. applied *in vivo* imaging to study the neocortex from 2.3–5 months old mice and found that newly formed spines possess functional synapses if they survived for a minimum of 4 days [235,236].

The degree of plasticity of spines is not only varying across age but also across brain regions, which may reflect that the functional needs for information reception and processing vary by age and by brain region, as will be discussed further[205,215,218].

1.3.3 Developmental and experience-dependent structural plasticity

Currently, our knowledge regarding activity-dependent structural changes in the spine is expanding ever more, especially in combination with the powerful tool of *in vivo* two-photon microscopy. By incorporating a variety of paradigms such as sensory deprivation, motor skilled learning, contextual and spatial learning, and so forth, researchers explored the experience-dependent spine modification in multiple cortical areas, including the primary somatosensory cortex (S1), the primary motor cortex (M1), the visual cortex (V1), and the hippocampus. In addition, studies are focusing not only on juvenile brains but also on adult, aging brains, as well as under both healthy and pathological conditions.

Holtmaat et al. and Trachtenberg et al. compared spine plasticity in S1 at 2 weeks, 2 months, 3 months, and 6 months of age, and found that the proportion of persistent spines (lifetime $\geq$ 8 days) increased from development to adulthood. Spine densities decrease during development while remaining relatively stable at 1 month, 2 months, and 6 months of age. Based on these findings, they point out that spines appear to progressively stabilize with age. In addition, they also compared the same cell type

(layer 5B pyramidal neurons) in S1 and V1 of 3- to 6-month-old mice and discovered a significantly higher spine turnover rate in S1 than in V1, suggesting that the capacity for spine remodeling during an experience may be different in these cortices[215,216]. In another publication, Holtmaat and co-workers introduced another sensory experience by trimming whiskers in a so-called chessboard deprivation way. Sensory input has then been changed and leads to a remodeling of synaptic connections by stabilization of experience-induced new spines as well as destabilization of pre-existing spines, besides, new-persistent spines tend to add more on the complex-tuft cells than on the simple-tuft cells in layer 5B of the barrel cortex[188].

In the visual cortex, Hofer and co-workers induced novel experience by introducing monocular deprivation (MD) through eyelid suture. During the first MD, spine formation of layer V neurons was markedly increased in the binocular cortex. Whereas, the spine density after the second MD did not show the same increase as after the first deprivation. In contrast, the ocular dominance (OD) shift was significantly enhanced after the second MD, suggesting that the ability to rapidly adapt to monocular deprivation can be generated not through the formation of physical connections but simply through the potentiation of pre-existing spines. However, it is noteworthy that the experience-dependent alteration in spine plasticity as described in layer 5 neurons has not been observed in layer 2/3 neurons in V1[226,237], probably owing to different mechanisms[226], which is presumably a lack of inhibition[237-239]. Indeed, Chen et al. observed that MD experience could induce significant inhibitory synapse loss both on shafts and on spines; the locally clustered inhibitory and excitatory synapses are coordinated with each other at the dendrite level and be influenced by experience. In this regard, excitatory synapse plasticity is influenced by the inhibitory synapse plasticity through suppression of calcium-dependent signaling events[237,240].

The activity-dependent spine plasticity has also been studied in M1. Xu et al. studied the apical dendrites of LVPNs and discovered that spine formation began within 1h after the initiation of the new motor skill training task, the following training process selectively stabilizes the new spines formed during the learning acquisition phase. The rapid spine addition, however, is accompanied by pruning off the pre-existing spines,

which demonstrates a rewiring process of the synaptic connections in the neuronal circuity during motor learning. In contrast, those mice failing to master the new task displayed no indication of increased spine formation or elimination[227]. Similar results have been obtained by Yang and co-workers, who observed the new spines formation within two days after training a novel rotarod task. In addition, practicing a different but still novel motor task can continuously drive synaptic reorganization in the pre-trained motor cortex, however, this new motor skill has been encoded by different synaptic sets, indicating a different motor memory storage[221]. Furthermore, learning-induced spine formation is not haphazard. Fu et al. studied the distribution of newly formed spines within M1 upon a novel seed-reaching task, revealed that learning-induced spines are not uniformly dispersed along the dendrites, instead, they are emerged and are packed into clusters, which presumably encode and transmit similar information[241]. Newly formed clusters of spines are also not randomly distributed, as has been shown by their avoidance of existing stable spines, which suggested that nearby spines might share and compete for local subcellular resources[241,242].

Spine plasticity studies have also been conducted in the aged brain and age-related neurodegenerative disease. Mostany et al. studied the barrel cortex in the aged mouse and observed that spines were more abundant, highly dynamic, and the number of thin, weaker spines was increased[243]. They proposed that this is a maladaptive process as it contradicts an efficient and less redundant neural circuit that the experience-dependent refinement process aims for[243]. Harris et al. studied rat hippocampal neurons and compared the spine distribution between a naïve slice sample (P15) and a mature tissue slice (P60-P75), proposed that long-term potentiation (LTP) enabled absolute synaptogenesis in the developing brain, however, shifted to a homeostatic process in adults through enlargement of specific synapses, induction of nearby spine clusters, and refrainment of distant spine outgrowth, thereby balancing the total excitatory inputs in an energy- and resource-conserving way[242].

As dendritic spines play an important role in the signal transmission between neurons, their plasticity and structural changes would affect the functionality of neuronal networks, which mediate learning and memory, making it essential to investigate and

appreciate these modifications in detail[205,214]. Pathological changes in dendritic spines have been identified to occur in numerous diseases, including AD[186], FTD[244], prion diseases[245-248], Parkinson's disease (PD)[249,250], Huntington's disease (HD)[251-253]. Therefore, it is necessary to study pathological alterations in spine plasticity and density also in ALS, especially use in vivo imaging methods.

1.3.4 The application of two-photon imaging in awake, behaving mice

With advances in bioimaging technology, the study of spine morphology has progressed considerably. Two-photon imaging allows us to observe deep brain tissue in real-time and non-invasively. The combined mode-locked Ti: Sapphire laser can be finely tuned the excitation light wavelength from 700nm to 1050nm for respective fluorophores, such as eGFP and its variants. This imaging method can enable a spatial resolution up to ~500 nm in the x/y axis[254]. A subset of neurons of interest can be tagged with fluorophores transgenetically or by viral transduction, thus enabling us to monitor the spine modification process upon experience.

Two-photon imaging has its unique advantage, especially compared to the conventional single-photon confocal imaging method: The laser used in two-photon imaging provides high-intensity pulses, which facilitate the simultaneous excitation of a fluorophore by 2 photons in a very circumscribed area. This process hence strongly reduces phototoxicity, out of focus excitation and the longer wavelengths enable deeper tissue penetration. These features render it better suitable for chronic *in vivo* imaging[255-257].

Most structural *in vivo* studies in the past have been conducted under anesthetized conditions to minimize brain displacement during data acquisition. Anesthetics inevitably have potential effects on synaptic plasticity and the overall neural activity in mice[258-260]. For example, isoflurane, a commonly used anesthetic, has been shown to inhibit excitatory synaptic transmission via presynaptic mechanisms[258]. Therefore, given the greater effects of anesthetics on brain activity, scientists have been working

over the recent years to image awake, motile animals[259].

In awake mice, the brain displacement associated with locomotion or behavior such as licking or grooming unavoidably affects the image quality, especially for subcellular structures at the micron level like spines[259]. To overcome the difficulties of imaging behaving mice we combine head-fixation and high speed data acquisition (frame rate of 30 Hz), to reduce within-frame movement artifacts. Mice are allowed to rest, groom, walk and run freely in both forward and backward directions on top of the air-supported styrofoam ball. Motion-related artifacts are later compensated by full-frame registration of the imaging data.

1.4 The hSOD1^{G93Adl} transgenic mouse model

1.4.1 Copy number determines disease phenotype in hSOD1^{G93A} transgenic mice

The establishment of multiple animal models derived from genetic mutations identified among fALS cases represents a valuable tool to investigate the pathogenesis of ALS disease. The first and most widely used rodent model is the hSOD1^{G93A} transgenic mouse model[58]. This model expresses high levels (~25 copies) of the human SOD1^{G93A} mutant transgene and develops focal paralysis at 3 months of age, progresses rapidly, and the mice die at 4-5- months old[58]. However, its aggressive disease course makes it not ideal to study early stages of the disease. Debates should not be neglected concerning whether the massive overexpression of the mutant SOD1^{G93A} protein is causing artifacts unrelated to the actual ALS pathology[261].

Recently, a hSOD1^{G93Adl} mouse model bearing only ~8-10 copies of the mutant gene was generated through the identification of mice with a spontaneous deletion of some of the SOD1^{G93A} transgene copies[261,262]. This mouse line displays a slower disease progression rate, in which symptoms commonly develop at ~7 months of age, and reaches the end-stage of life ~10 weeks after symptom onset[263,264]. Life expectancy of mice was extended from ~132 days to ~251 days because of the reduced expression

level of the mutant SOD1^{G93A} protein[265]. The hSOD1^{G93Adl} mouse model is thus more suitable to study the pathological mechanisms during the pre-symptomatic stage and to explore preventive treatment approaches[261,262,266]. Furthermore, Gurney et al. showed that the transgenic hSOD1^{G93Adl} mouse model is similar to human ALS in terms of pathological characteristics[265].

1.4.2 Histopathological features of the hSOD1^{G93Adl} transgenic mouse model

Histopathological changes in hSOD1^{G93Adl} transgenic mouse models have already been reported in several studies, occurring before the appearance of clinical symptoms. Jaarsma and co-workers first identified dot-like accumulations of mutant SOD1 from the brainstem motor nuclei as well as the spinal cord in the 3- to 5-week-old hSOD1^{G93Adl} transgenic mice, and the aggregates gradually increased in size and developed into vacuole-like structures with age[264]. In addition, they also found that the distribution, size, and emergence of the SOD1 immunoreactivity coincided with swollen mitochondria and the appearance of vacuolization in the axons and dendrites of motoneurons and that mitochondrial swelling was strongly associated with the aggregation of mutant SOD1 in their intermembrane spaces[264]. Puttaparthi found that the proteasome activity was reduced by 50% in the spinal cord of the 3-month-old hSOD1^{G93Adl} transgenic mice[267]. Additional molecular and morphological changes that precede spinal motor neuron death in hSOD1^{G93Adl} mice comprise several injury-related transcription factors expressed in a subpopulation of the spinal motor neurons at 15 weeks old, which include ATF3 (transcription factor 3), phospho-c-Jun, and CHOP (C/EBP homologous protein)/GADD153 (growth arrest- and DNA damage-inducible gene 153)[268], and their expression has also been detected in spinal interneurons at 20 weeks of age[268].

Fragmented Golgi apparatus has been detected in the spinal cord at 15 week-old[269]. The expression level of the heat shock protein 25 (Hsp25) (rat and human ortholog, heat shock protein 27 (Hsp27)) in the spinal motor neurons has been found to be

significantly reduced from 15 weeks of age onwards in the hSOD1^{G93Adl} mouse model[270]. Reduced fast and slow axonal transports have been found to occur in 200-day-old hSOD1^{G93Adl} mice, accompanied by neurofilament (NF) inclusions in vulnerable spinal motor neurons[271]. However, whether these axonal pathological findings develop at an earlier time remains to be further investigated[132]. Maatkamp et al. found the motor neuron loss and gliosis starting as early as 20 weeks of age, with less than 20% reduction in spinal motor neurons in 20-24 week-old hSOD1^{G93Adl} transgenic mice and 10%-60% loss in 25-29 week-old mice[270]. At 24 weeks of age, along with the reduction in spinal motor neurons, a significant reduction in the motor units of the extensor digitorum longus (EDL) muscles was observed; moreover, at a later stage of the disease, which is 34 weeks onwards, an asymmetric reduction of motor units of the EDL muscles was found on both sides of the mice[262].

However, cortical pathology with regard to the spine dynamics has never been studied in this mouse model and is thus the main objective of my thesis.

2. Objectives

With this study I aimed to characterize dendritic spine impairments of LVPNs in M1 in presymptomatic hSOD1^{G93Adl} transgenic mice *in vivo*, to address the following main questions:

1.) Are the density and the plasticity of spines on LVPNs affected in hSOD1^{G93Adl} transgenic mice?
2.) How rapidly is structural plasticity occurring *in vivo* in behaving mice? And does this short-term plasticity differ between CTR and hSOD1^{G93Adl} mice?
3.) Is motor skill learning impaired in hSOD1^{G93Adl} mice?
4.) Is structural plasticity associated with motor skill learning preserved in hSOD1^{G93Adl} mice?

3. Materials and Methods

3.1 Mice

hSOD1^{G93Adl} (B6.Cg-Tg (SOD1*G93A)^{dl}1Gur/J; *https://www.jax.org/strain/002299*) mice were initially obtained from the Jackson Laboratory and were subsequently crossbred with GFP-M transgenic mice (B6.Tg (Thy1-eGFP) MJrs/J, the Jackson Laboratory, *https://www.jax.org/strain/007788*), in which a subset of cortical LVPNs express eGFP under the control of Thy1 promoter[272]. The hSOD1^{G93Adl} × GFP-M transgenic mice (hereafter, SOD) were maintained on a C57BL/6J background by mating hemizygous transgenic hSOD^{G93Adl} with homozygous eGFP in our animal facility. Littermates that expressed eGFP, but not the hSOD1^{G93A} transgene were used as controls (hereafter, CTR). Animals employed in this study are from F_4-F_7 generations. Age and sex-matched 3 to 5 littermates were group-housed at our animal facility and were kept on a 12/12hr day/night cycle. Food and water were accessed *ad libitum*. All experiments are approved by the Government of Upper Bavaria (protocol number 11-16) and were reported in accordance with the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines[273]. Mice of both sexes were included in the SOD (7 females, 6 males) and CTR groups (3 females, 4 males). The mean ± s.e.m age at the first imaging time point for the SOD group is postnatal day 165.00 ± 3.24, and for the CTR group is 163.00 ± 5.00. The mean ± s.e.m age at surgery for the SOD group is postnatal day 136.56 ± 3.74, and for the CTR group is 133.83 ± 4.31.

3.2 Disease symptom screening

As in previous longitudinal experiments, this study has adopted regular and rapid assessments to monitor the symptoms of disease onset[261,274]. I used a battery of tests to assess disease symptoms twice a week, including the hind limb tremor test, hind limb extension test, tail elevation test, and rearing behavior test (Table 2)[275,276].

The criteria for euthanasia or termination in this experiment include up to 15% weight

loss over 48h, a behavior score > 6, behavior score > 1 longer than 48h or behavior score > 0 longer than 72h, self-mutilation, loss of the cranial window, not being accompanied by other littermates in the same cage or the end of the experiments for a given mouse.

Table 2. ALS score.

Parameter	Grade	Score
Hind limb tremor	Not present	0
	Present	1
Hind limb extension	Complete extension	0
	Partial extension	1
	No extension	2
Tail elevation test	At or above the level of the body	0
	Tail drops frequently	1
	No elevation	2
Rearing behavior test	Full extension of hind limbs at least twice in 5 min	0
	Full extension of hind limbs at least once in 5 min	1
	Incomplete or no extension of hind limbs	2

3.3 Surgery

The window implantation was conducted as previously described[202]. In brief, adult mice aged 4.5 months for both sexes were deeply anesthetized with a mixture of Medetomidine (0.5 mg/kg, Dormitor, Orion Pharma) - Midazolam (5 mg/kg, Dormicum, Roche) - Fentanyl (0.05 mg/kg, Hexal) intraperitoneally. The depth of anesthesia was checked by the absence of a toe-pinch reflex as well as a slower, steady respiratory rate. The mice were subsequently kept at 37°C by placing them on a heating pad and carefully positioned into a stereotaxic instrument. To expose the skull, the fur was excised with sterile surgical scissors. The periosteum was removed with a scalpel blade to help with the adhesion of the head post. The right M1 was located and marked based on stereotactic coordinates. A circular craniotomy overlying the right M1 (coordinates

of the center of the cranial window: from bregma anterior-posterior (AP), 0.3 mm; medio-lateral (ML), 1.7 mm) (Fig. 1D)[227,277,278] was performed using a high-speed surgical drill, great precaution has been taken during drilling to avoid potential bleeding due to damage of the vasculature, however, if it occurred, sterile saline (B. Braun, Melsungen, Germany) and gelfoam (Medline) were applied to stop the bleeding. Once the bone flap overlying the right M1 became loose, it was gently lifted and removed through insertion into the gap between the bone flap and the dura with fine forceps, while leaving the dura intact. To prevent the cortex from drying, sterile saline was periodically added. Once the bleeding had ceased by the application of sterile saline and wet gelfoam, a glass coverslip (4mm in diameter, Warner Instruments) was implanted and properly adjusted to fit into the cranial window. With sterile surgical sponges, sterile saline was soaked up to create a dry surface to facilitate the initial seal between the coverslip and the skull. Subsequently, small amounts of UV curable dental cement (Venus Diamond Flow), histoacryl tissue glue (B. Braun, Melsungen, Germany), and dental acrylic (Paladur) were used to help with the affixation of a custom-designed aluminum head post, which supports the head fixation under the objective for the following imaging sessions. Animals were returned to their home cage for 3-4 weeks to allow for recovery from the surgery before imaging experiments started. Analgesia (Metacam, 1mg/kg) has been administered orally 30min before surgery and every 24h post-surgery for 72h in accordance with the animal licenses.

3.4 Two-photon structural imaging in awake, behaving mice

In this study, I chronically followed the spine dynamics of LVPNs in M1 under baseline conditions and during a motor skill learning paradigm. An experimental timeline is shown in Fig. 1A. Mice were mounted under a two-photon microscope equipped with a mode-locked Ti: sapphire laser (Mai Tai eHP, Spectra-Physics) tuned to 925nm for excitation. Then images of fluorescently labeled pyramidal neurons were acquired on a resonant scanning (frame rate: 30Hz) two-photon microscope (Hyperscope, Scientifica), using a 16x, 0.8 numerical aperture objective (Nikon). To mount the mice on a spherical

treadmill underneath the objective, they were anesthetized with 3% isoflurane (Abbott, Chicago, Illinois, USA) for 2-3min. To get accustomed to the air-supported spherical treadmill, mice had been trained for 3 sessions over 6 hours in total before the start of the regular experiment. Well-trained mice would move freely on the spherical treadmill without signs of distress (Fig. 1B). Vasculature patterns were used as landmarks for the identification and relocation of imaging sites in each imaging session. Image stacks capturing the apical dendrites of interest within 100μm below the pial surface were acquired at a resolution of 512 × 512 pixels (covering 97 × 97μm), and with 100 frames per plane, 40-110 planes per mouse, 1μm z-increment between planes. Overview image stacks (512 × 512 pixels, covering 700 × 700μm, 5μm z-steps) that captured the dendrites all the way to their respective soma (525-700μm in depth) were acquired to identify LVPNs. Similarly, detailed coordinates of the dendrite of interest with high magnification were stored with respect to fiduciary points in low magnification images (Fig. 1C), which had been set as landmarks in previous imaging sessions. The laser power was ~10-20mW to sustain similar fluorescence levels across multiple imaging sessions. To follow spine dynamics under baseline conditions and during a motor skill learning task, the same dendrite of interest was imaged over 7 imaging sessions (every other day). Moreover, during each imaging session in awake mice, the same dendrite and its spines were captured three times at 50 min intervals, during which the mice were allowed to run freely on the spherical treadmill, and their walking or running behavior was recorded by an optical mouse sensor to gain insight into short-term spine plasticity. To investigate whether the novel wheel running skill will leave a prolonged memory trace and to explore the survival fraction of the new spines formed during the motor learning phase, spines were imaged after 11 repeated training sessions on the complex wheel and again 1-month post-training in a subset of animals (Fig. 1E-F). Although mice were moving freely on the spherical treadmill, image acquisition was avoided as much as possible while mice were running, but rather while they were resting, grooming, or walking slowly. In addition, to remove motion-related lateral shifts, imaging stacks were registered by custom-written routines in Matlab[279].

3.5 Image analysis

Dendritic spines were analyzed manually using ImageJ (*http://rsbweb.nih.gov/ij/*) according to established quantification criteria[202]. To be more specific, protrusions emanating laterally from the dendritic shaft (> 0.4 μm) were analyzed as spines. Spines regardless of their morphological subtypes were included in this study. All spines were counted by the experimenter being unaware of the genotype of the respective animals and their performance in the wheel training. Images were aligned using fiduciary points which are consistent throughout all imaging sessions e.g. dendritic branch patterns. Spines are tracked in three-dimensional stacks to ensure the identification of spines being not affected by potential tissue movements or any rotations. Spines were considered as stable if their positions with respect to adjacent landmarks were within 0.5 μm in consecutive image time points. Formation of a spine was well-defined as a spine that emerged at a location with no apparent protrusion during the last time point, whereas elimination of a spine was well-defined as the absence of one at a location where a spine was visible at the previous time point. The spine density of randomly selected dendritic segments was analyzed for both genotypes. The turnover rate of spines was calculated as TOR (t1, t2) = $(N_{gained} + N_{lost})/ (N_{t1} + N_{t2})$, where N_{gained} is the number of new spines, N_{lost} is the number of lost spines, N_{t1} and N_{t2} are the numbers of total spines present in the first image and the second image, respectively. I also computed the survival fraction of individual spines, which was calculated as SF (t) = N_t/N_0, where N_0 is the number of spines at t = 0, and N_t is the number of spines that still existed after time t.

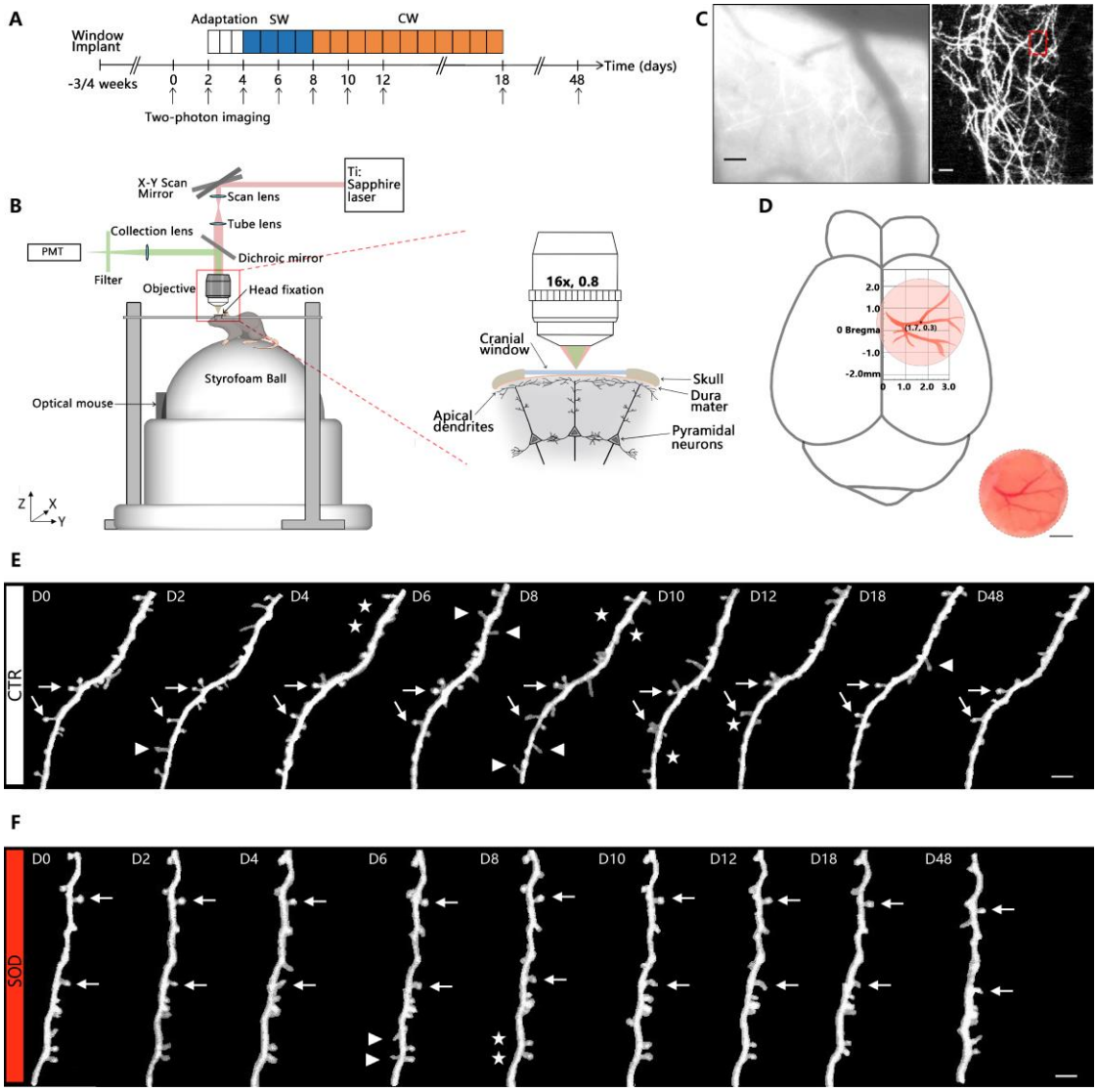

Figure 1. Experimental design and representative images under baseline conditions and during a motor skill learning task.

(A) Timeline of experiments. The adaptation phase (indicated in white box) consisted of imaging once in the absence of a wheel in the chamber and twice in the presence of a locked wheel placed in the chamber. The blue box indicates the standard wheel training session; the orange box indicates the complex wheel training session. (B) A schematic diagram of the two-photon microscope apparatus applied to structural imaging of an awake, behaving mouse. The mouse is placed on top of a styrofoam ball, which is supported by pressurized air, allowing the mouse to rest, walk or run freely. The mouse's head is fixed on the head-plate during imaging (left). The magnification displays the optical access to the neurons and their dendrites/spines in cortical layer I of M1 through the cranial window (right). (C) An image of the vasculature pattern of M1 was acquired with a CCD camera (left). A low magnification view of the dendrites was imaged with a two-photon microscope (right). (D) A scheme of the window location and imaged brain area (upper left). The photograph on the right shows a representative implanted window after the operation (lower right). (E) A high-magnification view of the dendrite in the boxed area (red) in C is shown. Representative images from a CTR mouse across the experimental period. (F) Representative images from a SOD mouse.

Arrowheads refer to the spines newly formed; Asterisks mark locations in which spines have disappeared; arrows denote stable spines that are present throughout the entire imaging series. SW, standard wheel; CW, complex wheel. Scale bars: 20μm in C, 1mm in D, 5μm in E and F.

3.6 Behavioral tasks

3.6.1 Open field test

As a necessary step to familiarize the mice with the chamber environment, an open field test (OFT) was performed during the habituation sessions. This exploratory task is probing a number of different parameters, such as exploration and locomotor activity, and also to some extent avoidance behavior and anxiety-like behavior[280-282]. Mice naturally avoid the center area of the open-field arena and will explore the corners and areas close to the walls more frequently[281,283-285]. Locomotion within the open-field arena was measured in a plexiglas chamber with a length, width, and height of 40 × 40 × 40 cm, respectively. Approximately 65% area in the center of this open field arena is defined as the center area (32cm × 32cm). The walls of the chamber are covered by white styrofoam and the experimenter is leaving the immediate surroundings such that mice cannot see the experimenter, in order to avoid unnecessary agitation and stress to the mice. The ambient temperature and light intensity of the behavioral testing room were similar to those of the home cages and met the criteria of the Upper Bavarian Government for behavioral experiments on mice. Every mouse has been tested individually once for 30 min. A camera was positioned directly above the open field to ensure that the entire trajectory of the mouse in the chamber could be captured. When the experiment was completed for each mouse, the floor was cleaned so that the chamber could then be used for the next mouse.

The following behavior parameters were scored and analyzed using the plugin MouBeAT from ImageJ (*http://rsbweb.nih.gov/ij/*): the total distance traveled, the average speed, number of movements into the center, the total freezing time (defined as the time during which the animal was completely inactive, except for minor movements associated with breathing) [286,287].

3.6.2 Voluntary motor skill learning paradigm

After mice were familiarized with the open field arena and the locked-wheel (which was positioned in the central area of the open arena but blocked for 2 sessions (30-minute per session)), I started the motor skill training using the standard wheel (hereafter, SW). To this end, mice learned to maneuver our custom-designed running wheel placed in the center during daily training sessions (30 min, custom-designed wheel, diameter, 21.6 cm; circumference, 67.8 cm; rung distance, 2.1cm). The rungs of the wheel are regularly distributed (Fig. 2). Mice could freely explore the wheel (step in or out) as well as explore the open arena. An optical mouse sensor was attached to the wheel to detect its rotation. Data of the running performance were processed by custom software written in LabVIEW (National Instruments). The mice were returned to their house cages for further rearing after 30 minutes of free access to the wheel. Mice were exposed to the SW 30 min per day for continuous 4 days. After 4 SW training sessions, a different motor skill learning task was initiated by removing 3 rungs and loosening 4 rungs of the wheel at random locations, thereby adding complexity compared to the SW learning task (hereafter, complex wheel, CW, Fig. 2). Loosening the rungs is provoking a slipping through upon the positioning of a limb. Mice were then subsequently exposed to the CW for another 11 consecutive days the same way with the SW (Fig. 1A). The running activity pattern has been recorded with a camera in front of the training box and the optical mouse sensor positioned on the wheel. The impact of this motor skill learning paradigm on the structural plasticity of cortical neurons was assessed during repeated imaging at 2-day intervals (Fig. 1A). Parameters of wheel running were measured on a daily basis, including running distance, running duration, and maximum running speed.

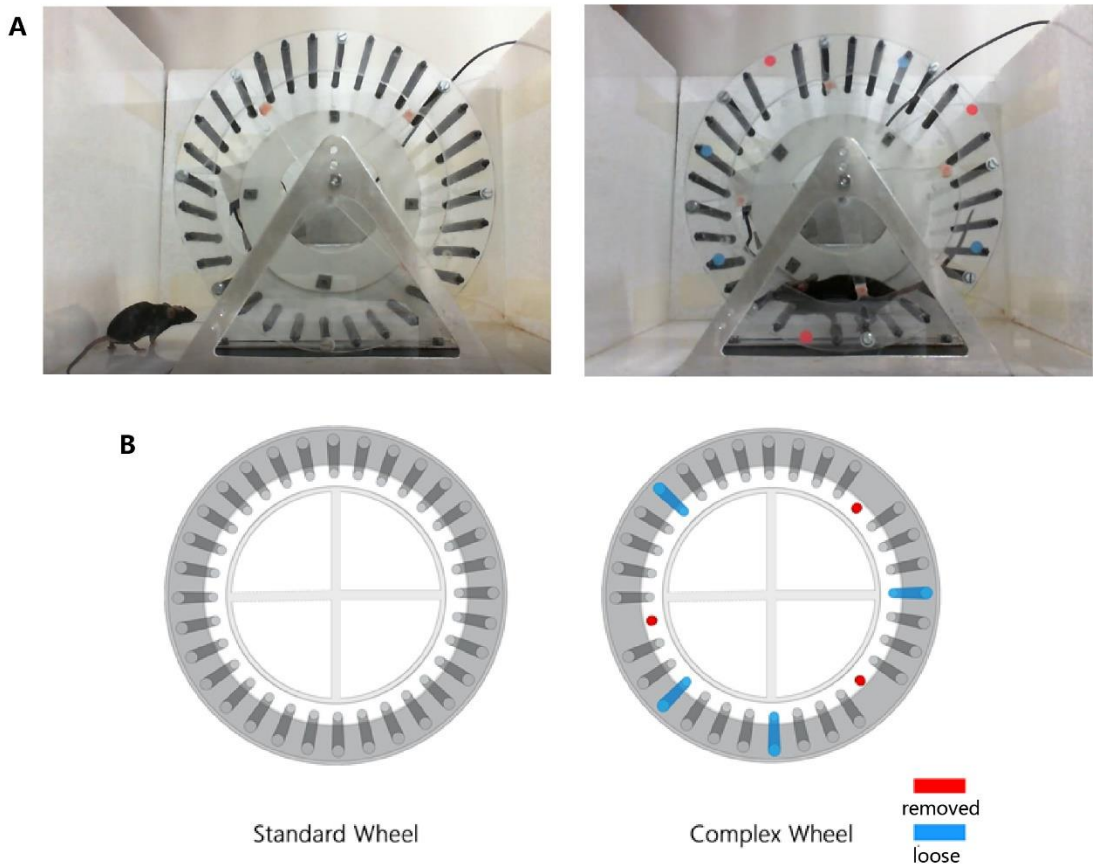

Figure 2. Design of the standard and complex wheel.
(A) Example images of the standard wheel (left) and the complex wheel (right). (B) Schematic diagrams of the design of the standard wheel and the complex wheel in this experiment. The standard wheel has 32 evenly spaced rungs; in the complex wheel 3 rungs were removed (indicated by red dots) and 4 rungs were loosened (indicated by blue dots).

3.7 Statistics

Statistical analyses in the present experiment were performed in MATLAB, SPSS, or GraphPad Prism. Data that are normally distributed were compared using a two-sided Student's t-test. Data that are non-normally distributed were compared using a non-parametric Mann-Whitney U-test. The statistical tests are reported in the respective result sections. Mouse running performance in the wheel was probed by a repeated measures (RM) two-way ANOVA. The overall difference in wheel running performance between the different strains of mice was indicated by p (m), and the divergence over time between the two groups was indicated by p (i). Correlations between spine modifications, including formation rate, elimination rate, and turnover rate, and the total distance of running on the 1st and 2nd day of SW training were

evaluated with Pearson's correlation coefficient (r). While correlations between short-term spine modifications, including formation rate, elimination rate, and turnover rate, and running distance on the styrofoam ball were analyzed by Spearman's correlation coefficient (r). Data are presented as mean ± s.e.m for normally distributed data or median with interquartile range (IQR) for non-normally distributed data. Statistical significance is indicated by *p < 0.05; **p < 0.01; ***p < 0.001. The *n* in Results 4.2 reported in the text represents the number of mice, and the remaining represent the number of dendrites.

4. Results

4.1 Presymptomatic hSOD^{G93Adl} mice display altered spine density and dynamics under baseline conditions

To characterize both long and short-term structural plasticity of dendritic spines of LVPNs, I repeatedly tracked and analyzed dendritic segments of their apical tufts within layer 1, that is, within 100μm from the brain surface. Images were acquired every 50 minutes to assess short-term plasticity, and every other day over 2 weeks to assess long-term spine alterations.

Spine density in the pre-symptomatic SOD mice was significantly reduced compared to the CTR mice (CTR, 0.47 ± 0.02 μm^{-1}, n = 13; SOD, 0.36 ± 0.01 μm^{-1}, n = 13; p < 0.001; Student's t-test, Fig. 3A).

Subsequently, the formation rate and elimination rate of spines were calculated separately, which indicated that both the formation rate and the elimination rate in SOD mice were lower than those in the CTR group (formation rate (two-day interval): CTR, 0.15 ± 0.02, n = 13; SOD, 0.07 ± 0.01, n = 13; p < 0.001; Student's t-test, Fig. 3B; elimination rate (two-day interval): CTR, 0.20 ± 0.02, n = 13; SOD, 0.11 ± 0.01, n = 13; p < 0.001; Student's t-test, Fig. 3C). Furthermore, the spine turnover rate of SOD mice was remarkably lower than that of CTR mice over long-term interval (turnover rate (two-day interval): CTR, 0.18 ± 0.01, n = 13; SOD, 0.09 ± 0.01, n = 13; p = 0.007;

Student's t-test, Fig. 3D).

The survival rate of the spines was calculated as a function of time by tracking and analyzing the lifetimes of the individual spines where observed during the first time point. I found a significantly higher fraction of spines in the SOD group still being present after 4 days compared to the CTR group (CTR, 0.74 ± 0.02, n = 13; SOD, 0.83 ± 0.02, n = 13; p = 0.003; Student's t-test, Fig. 3E).

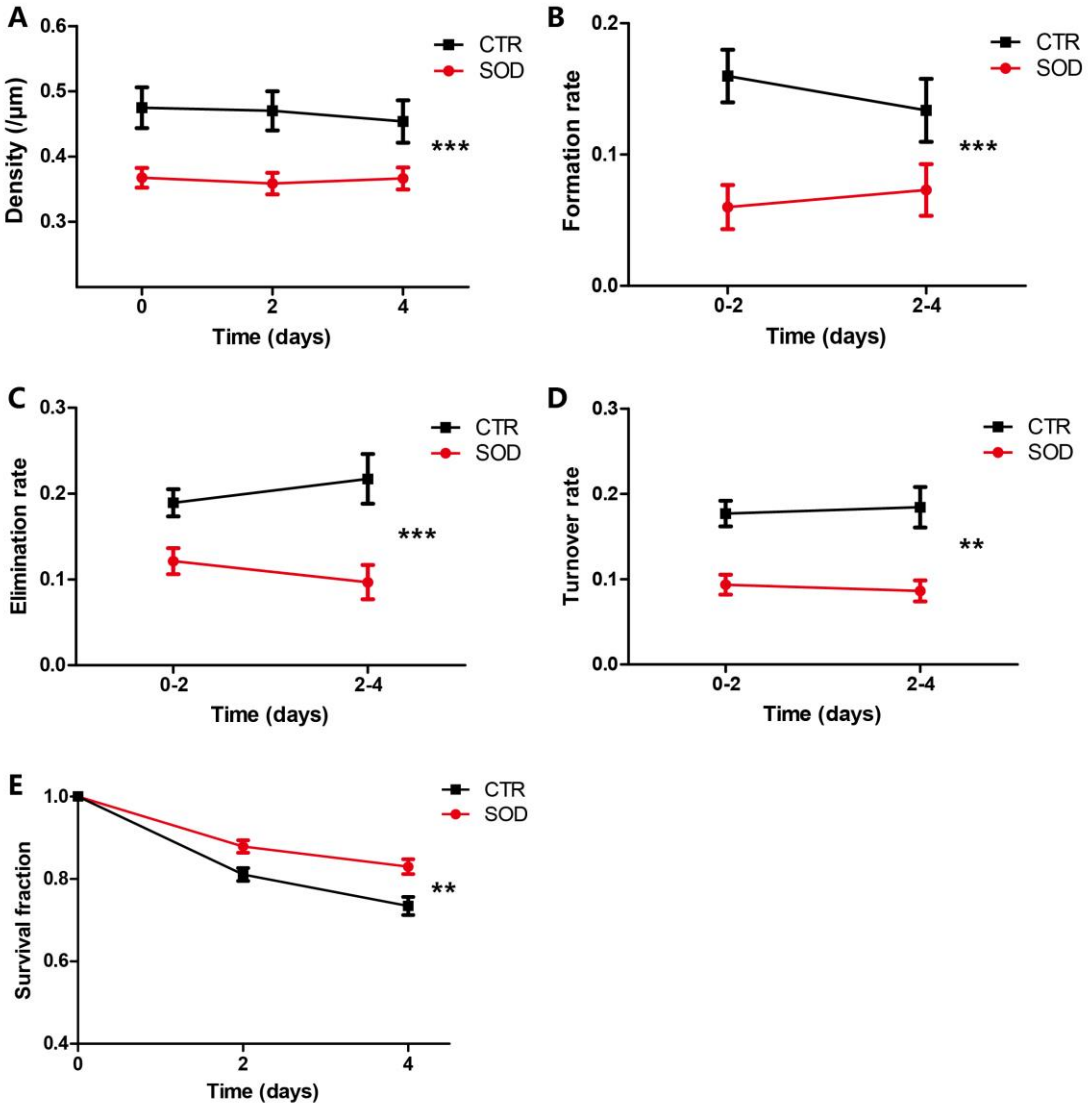

Figure 3. Spine modifications under baseline conditions.

(A) Spine density was significantly reduced in SOD mice. (B) The spine formation rate, (C) elimination rate, (D) and consequently the spine turnover rate at 2 days interval was reduced in the SOD mice. (E) The survival fraction of spines over 4 days was higher in the SOD group than that in CTR mice. Data are indicated as mean ± s.e.m. **p < 0.01, *** p < 0.001.

4.2 Presymptomatic hSOD^{G93Adl} mice display reduced exploratory behavior and locomotion in the open-field

After 3 awake imaging sessions on the spherical ball, to assess the locomotor and exploratory behavior of SOD transgenic mice, I recorded their behavior in an open field test over 30 min (Fig. 4A-B).

SOD mice traveled a significantly shorter distance compared to the CTR group over 30 min (CTR, 13792.03 ± 1084.67cm, n = 7; SOD, 8105.17 ± 780.01cm, n = 13; $p < 0.001$; Fig. 4C), and also exhibited a significant reduction in the average speed in comparison to their littermate group (CTR, 7.67 ± 0.58cm/s, n = 7; SOD, 4.62 ± 0.42cm/s, n = 13; $p < 0.001$; Fig. 4D). SOD mice also entered into the center area significantly less compared to CTR mice (CTR, 214.43 ± 23.05, n = 7; SOD, 102.54 ± 13.38, n = 13; $p < 0.001$; Fig. 4E). Additionally, in comparison to the littermate group, the SOD transgenic mouse group displayed a remarkable increase in total freezing time (CTR, 793.50 ± 122.96s, n = 7; SOD, 1184.26 ± 29.81s, n = 13; $p < 0.001$; Fig. 4F).

These exploratory deficits were not due to actual motor symptoms, as the SOD score of these presymptomatic mice was zero. Furthermore, to overcome the limitations of the subjective assessment of the SOD score, I assessed their motor performance on a running wheel (the standard wheel and the complex wheel running task) (see Results 4.4).

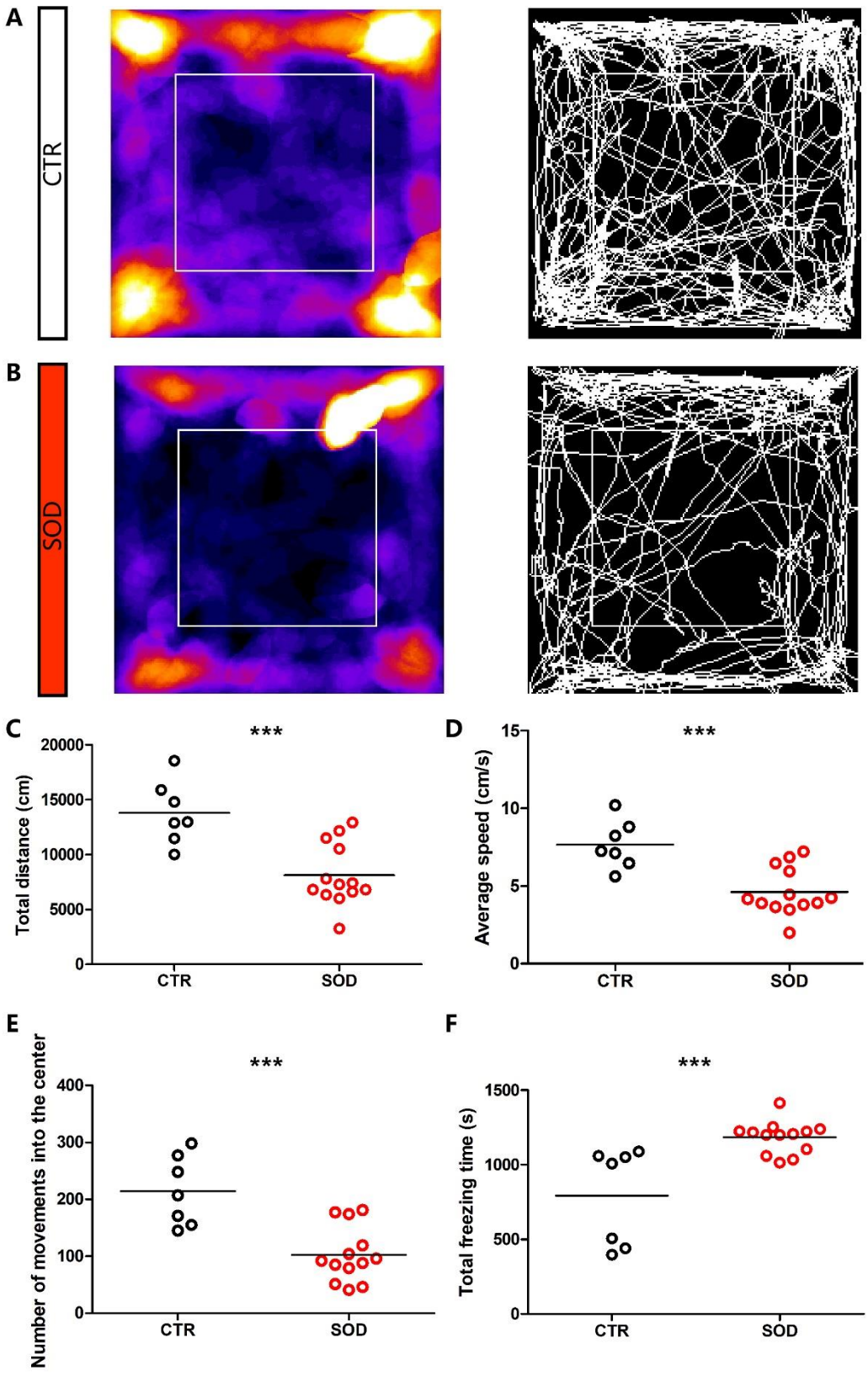

Figure 4. Open field test.

(A-B) Representative open field locomotion heatmaps (left) and trajectories (right) of 10 minutes for CTR mice (A) and SOD mice (B) are shown respectively. (C-F) The following four parameters were analyzed: the total distance traveled (C), the average running speed (D), the number of

movements into the central area of the open arena (E), and the total freezing time (F). The SOD mice showed a decreased exploratory behavior compared to the CTR mice. Data points represent individual mice and are superimposed by the mean. ***p < 0.001.

4.3 Short-term structural plasticity and the impact of locomotion on the styrofoam ball

I further followed short-term spine plasticity and explored the impacts of locomotion on the styrofoam ball upon the plasticity of the spines. The spine turnover rate of SOD mice was markedly lower over a 50-minute short-term interval compared to the CTR group (turnover rate (50-min interval): CTR, 0.05 ± 0.004, n = 13; SOD, 0.03 ± 0.003, n = 13; p < 0.001; Student's t-test, Fig. 5). Representative images regarding short-term spine modifications are shown in Figure 6.

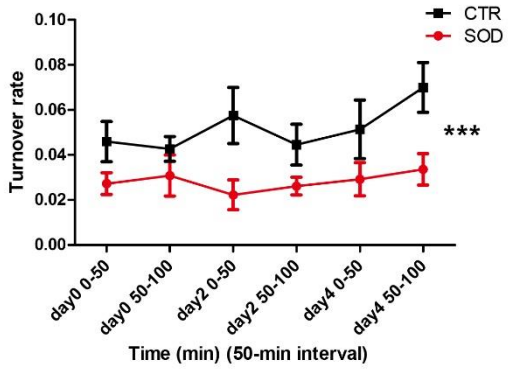

Figure 5. Short-term spine turnover rate.
In comparison to the CTR group, SOD mice had significantly lower turnover rates at 50-minute short-term intervals. Data are indicated as mean ± s.e.m. *** p < 0.001.

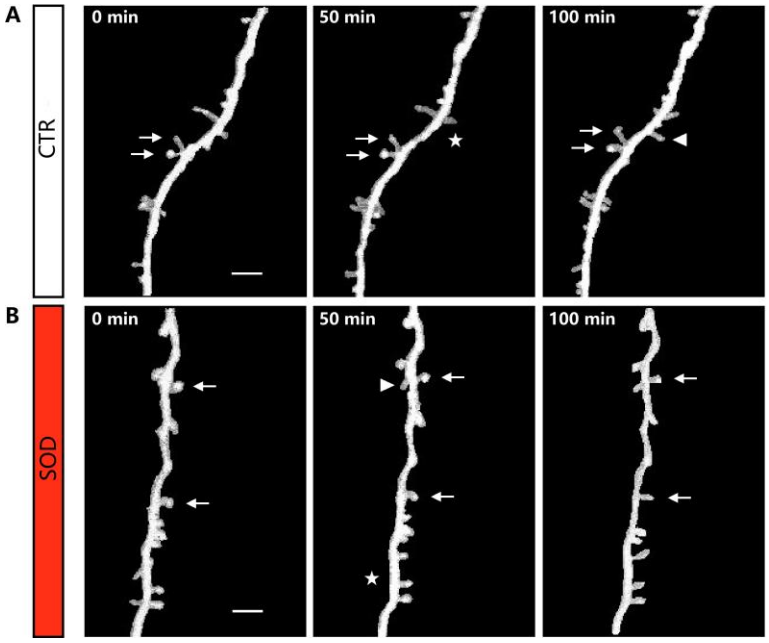

Figure 6. Short-term spine modifications.

(A) Representative images from a CTR and (B) a SOD mouse over two 50 min short-term intervals. The arrowheads refer to newly formed spines; the asterisks refer to the location of a disappeared spine; the arrows represent stable spines that are present throughout the entire imaging series. Scale bars: 5µm.

Short-term spine modifications were further investigated with respect to the behavior performance on the styrofoam ball. No significant correlation was detected between the short-term formation rate of spines and the running distance in the CTR group (Spearman's correlation coefficient r = 0.14, p = 0.07, Fig. 7A) and the SOD group (Spearman's correlation coefficient r = 0.10, p = 0.18, Fig. 7B). However, a weak positive correlation was found between the short-term elimination rate of spines and running distance in the CTR group (Spearman's correlation coefficient r = 0.17, p = 0.03, Fig. 7C), but not for SOD mice (Spearman's correlation coefficient r = 0.10, p = 0.16, Fig. 7D). Moreover, weak but positive correlation also exists between the short-term turnover rate of spines and running distance in CTR mice (Spearman's correlation coefficient r = 0.21, p = 0.01, Fig. 7E), but not in SOD mice (Spearman's correlation coefficient r = 0.12, p = 0.10, Fig. 7F).

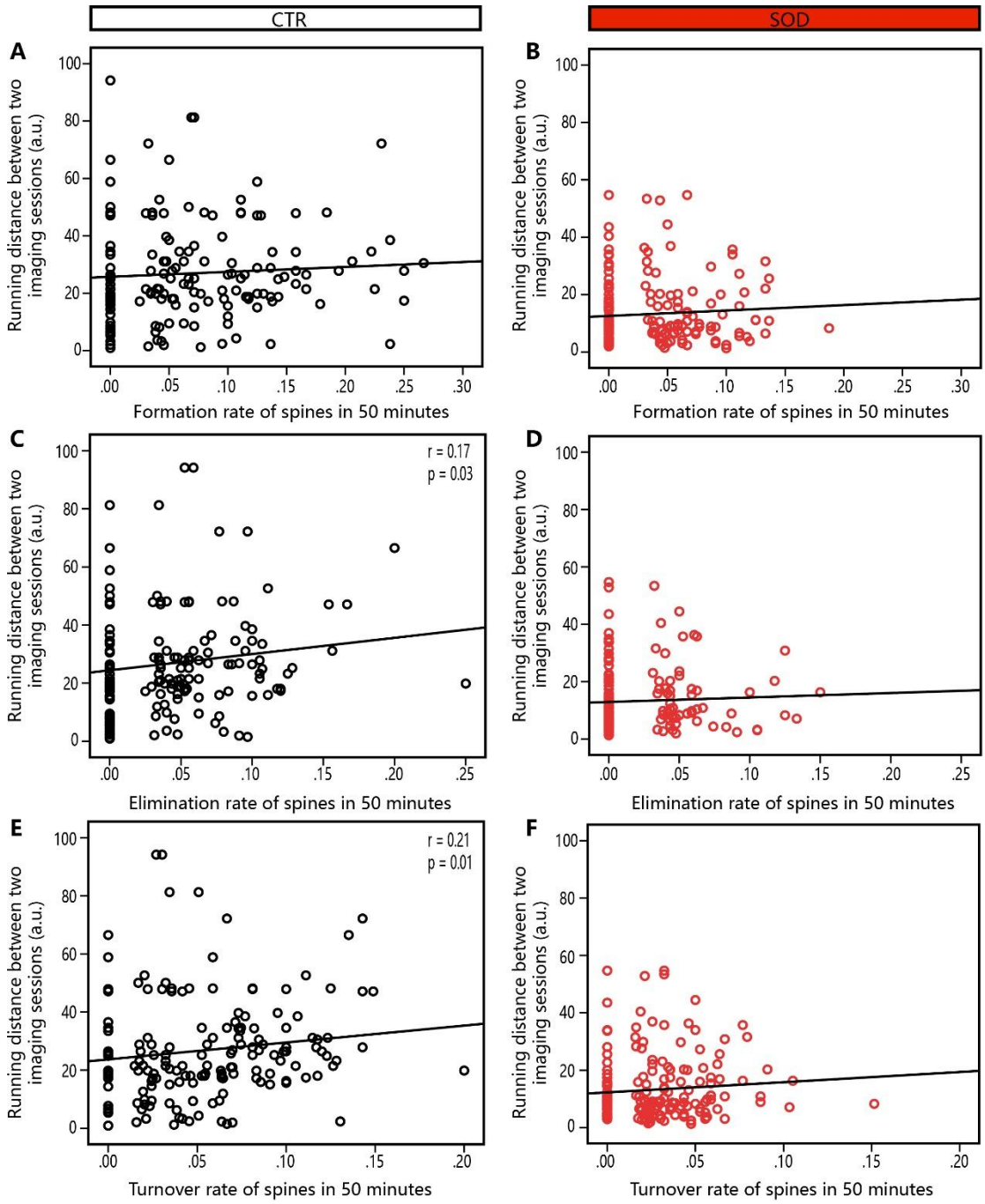

Figure 7. Short-term spine modifications correlate with behavioral performance.
(A-B) The short-term formation rate of spines did not correlate with running distance in the CTR group and the SOD group. (C-D) However, the short-term elimination rate of spines was positively correlated with running distance in CTR mice, but not in SOD mice. (E-F) The short-term turnover rate of spines was positively correlated to running distance in the CTR group, but not in the SOD group. r is Spearman's correlation coefficient. a.u., arbitrary unit. Statistical significance is determined by $p < 0.05$.

4.4 Performance in the motor skill learning task

After the OFT, the mice were exposed to a voluntarily running wheel test. Mice were first exposed to an SW running test for 4 consecutive days over 30 minutes per day, followed by a CW running test for 11 consecutive days also for 30 minutes per day (see Methods). Mice usually run spontaneously on the wheel within a few minutes upon being provided with the wheel. Mice quickly mastered the wheel and finally reached a performance plateau [288]. After 4 days of SW training, a CW training was introduced, where the mice needed to adapt the motor pattern to account for irregular spacings and loosened rungs, which caused the slipping of the limbs at unpredictable intervals.

Results showed that almost all mice would enter and run in the wheel within the first 1 or 2 days; only 1 mouse started to enter and run in the wheel after 7 days of exposure to the wheel and this mouse, which was defined as a non-learner, was excluded from the experiment. This experiment included 9 mice in the SOD group and 5 mice in the CTR group. The running performance of both the SOD transgenic mice and CTR mice improved gradually, and at around 11 days, the maximum running velocity and running distance plateaued in both groups (Fig. 8A-B). The maximum running velocities on both SW and CW was not significantly different between the SOD and CTR groups (genotype effect $F_{(1,173)} = 0.16$, p (m) = 0.69; time effect $F_{(14,173)} = 0.27$, p (i) = 1.00, two-way RM ANOVA, Fig. 8A). Furthermore, the distance traveled on both SW and CW was also not significantly different between the SOD group and the CTR group (genotype effect $F_{(1,173)} = 0.82$, p (m) = 0.37; time effect $F_{(14,173)} = 0.56$, p (i) = 0.89, two-way repeated measures ANOVA, Fig. 8B). These results indicate that the wheel running performance was similar between the two groups, and no deficits in motor activity and performance were observed between the two groups at the presymptomatic stage.

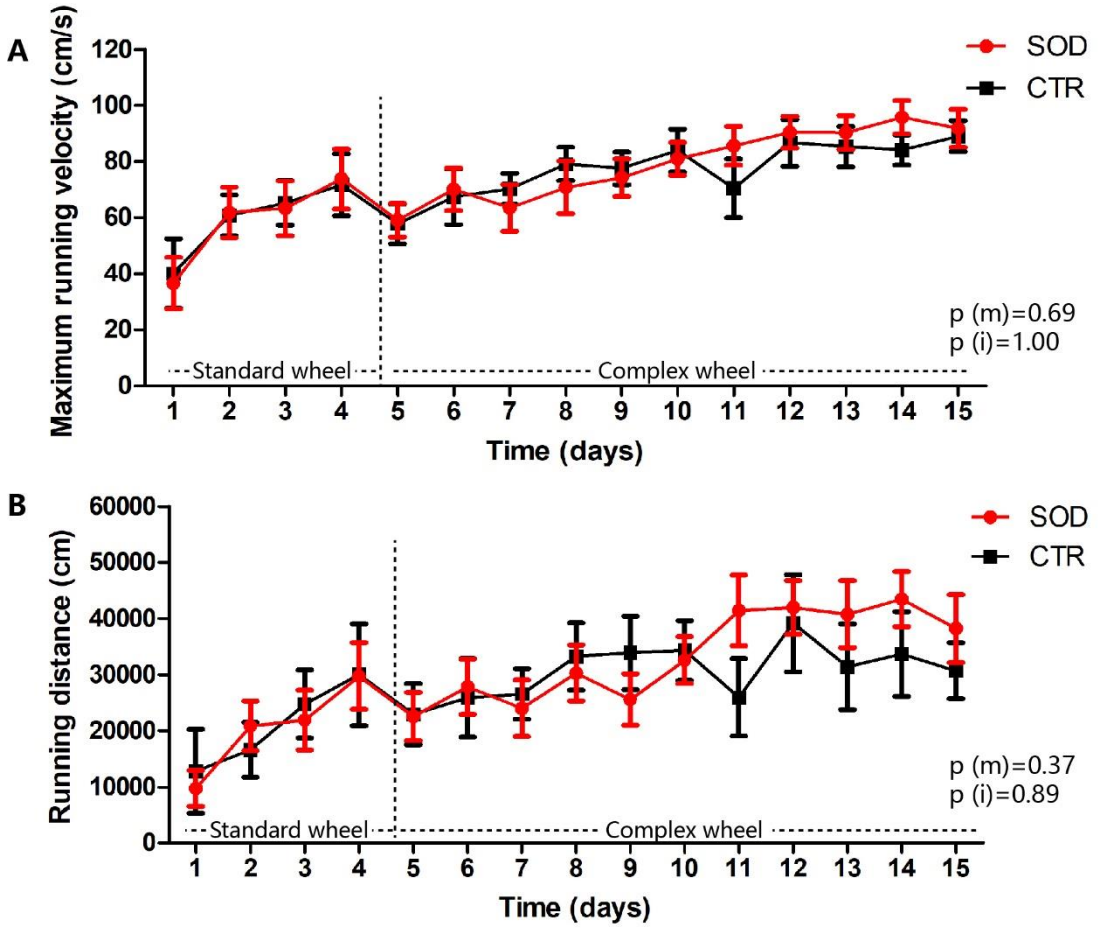

Figure 8. Running performance during exposure to the standard wheel and the subsequent complex wheel.

The running performance of both CTR mice (black) and SOD mice (red) gradually increased and finally reached a plateau. Maximum running velocity (cm/s) and running distance (cm) are shown in (A) and (B), respectively. Differences between genotypes are indicated by p (m), and the effect of time is indicated by p (i). Statistical significance is determined by $p < 0.05$. Data are indicated as mean ± s.e.m.

4.5 Impact of motor skill learning on structural plasticity

Through the motor skill learning process, I sought to explore whether voluntarily learning to run on the wheel would induce alterations in the dynamics of spines in M1 of both groups. The SW learning task induced an increase on spine formation rate significantly after 2 days in the CTR group compared to the baseline levels (formation rate (2-days of SW training): CTR, 0.21 ± 0.03, n = 12; baseline, 0.15 ± 0.02, n = 13; p = 0.04; Student's t-test, Fig. 9A), while for the SOD mice, although a slight increase

was also observed, it was not significantly different compared to baseline levels (formation rate (2-days of SW training): SOD, 0.07 ± 0.02, n = 13; baseline, 0.07 ± 0.01, n = 13; p = 0.73; Student's t-test, Fig. 9B). In addition, the absolute value of newly formed spines after 2-day SW learning were significantly lower in SOD group than in CTR group (CTR, 0.09 ± 0.01 μm^{-1}, n = 12; SOD, 0.03 ± 0.01 μm^{-1}, n = 13; p = 0.001; Student's t-test).

However, a slight increase in the formation rate of spines has also been observed in both CTR and SOD groups after two days of exposure to CW, although no observed significant difference in comparison to the baseline levels (formation rate (2-days of CW training): CTR, 0.19 ± 0.04, n = 12; baseline, 0.15 ± 0.02, n = 13; p = 0.25; Student's t-test, Fig. 9A; SOD, 0.09 ± 0.01, n = 13; baseline, 0.07 ± 0.01, n = 13; p = 0.19; Student's t-test, Fig. 9B)).

In addition, the elimination rate of spines did not differ from their baseline levels for both SOD and CTR groups after 2 days of SW learning, and for both groups after 2 days of CW learning (elimination rate (2-days of SW training): CTR, 0.21 ± 0.03, n = 12; baseline, 0.20 ± 0.02, n = 13; p = 0.74; Student's t-test, Fig. 9C; SOD, 0.14 ± 0.01, n = 13; baseline, 0.11 ± 0.01, n = 13; p = 0.15; Student's t-test, Fig. 9D; elimination rate (2-days of CW training): CTR, 0.22 ± 0.04, n = 12; baseline, 0.20 ± 0.02, n = 13; p = 0.71; Student's t-test, Fig. 9C; SOD, 0.09 ± 0.02, n = 13; baseline, 0.11 ± 0.01, n = 13; p = 0.38; Student's t-test, Fig. 9D)).

The turnover rate was also compared to the baseline levels after 2 days of SW training, and the results showed no difference in both SOD and CTR mice compared with their baseline levels, respectively (turnover rate (2-days of SW training): CTR, 0.21 ± 0.02, n = 12; baseline, 0.18 ± 0.01, n = 13; p = 0.19; Student's t-test, Fig. 9E; SOD, 0.11 ± 0.01, n = 13; baseline, 0.09 ± 0.01, n = 13; p = 0.16; Student's t-test, Fig. 9F)). Similarly, the comparison of the turnover rate after 2 days of CW training with baseline levels also showed no difference in both SOD and CTR group (turnover rate (2-days of CW training): CTR, 0.21 ± 0.03, n = 12; baseline, 0.18 ± 0.01, n = 13; p = 0.41; Student's t-test, Fig. 9E; SOD, 0.09 ± 0.01, n = 13; baseline, 0.09 ± 0.01, n = 13; p = 0.88; Student's t-test, Fig. 9F)).

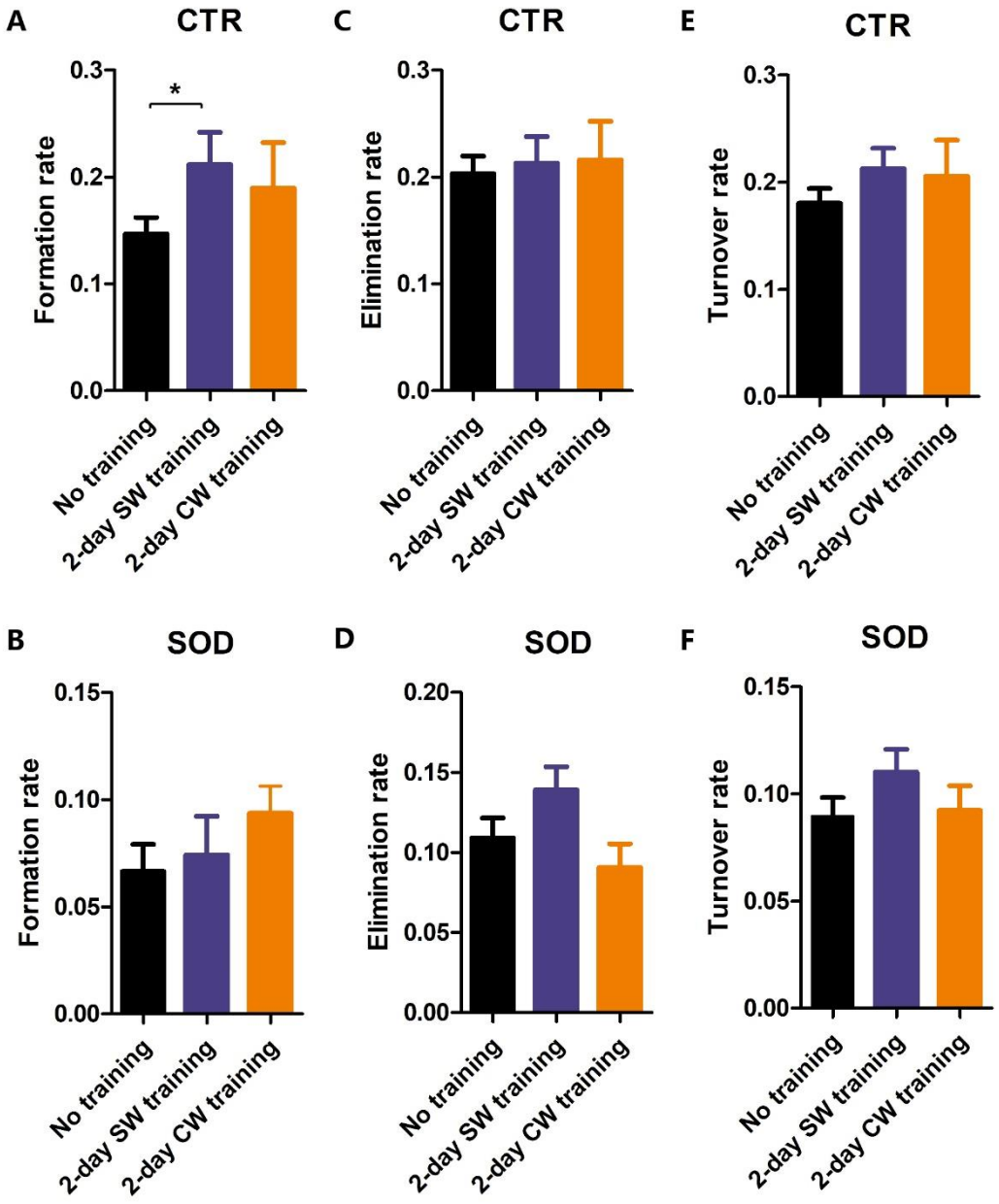

Figure 9. Experience-dependent spine plasticity after motor skill learning.
(A) SW learning resulted in an increase in spine formation in the CTR group, (B) while mice in the SOD group did not show such an increase. After 2 days of CW training, neither the CTR mice (A) nor the SOD mice (B) exhibited a rapid increase in spine formation rate in comparison to the pre-training period. In addition, after 2 days of SW training and 2 days of CW training, the elimination rate did not show any difference in both the CTR group (C) and the SOD group (D) compared to the pre-training period, respectively; similarly, the turnover rate of spines did not show any difference in both the CTR group (E) and the SOD group (F) compared to the pre-training period. Data are indicated as mean ± s.e.m. SW – standard wheel, CW – complex wheel, *$p < 0.05$.

I furthermore investigated the survival fraction of spines newly formed within 2 days of SW training, with no observed differences between the SOD group and the CTR

group (survival fraction after 4 days: CTR, 0.27 ± 0.08, n = 13; SOD, 0.23 ± 0.13, n = 10; p = 0.26; Mann-Whitney U-test; Fig. 10A; survival fraction after 12 days: CTR, 0.08 ± 0.04, n = 7; SOD, 0.22 ± 0.15, n = 9; p = 0.76; Mann-Whitney U-test; Fig. 10A; survival fraction after 42 days: CTR, 0.04 ± 0.04, n = 4; SOD, 0.00 ± 0.00, n = 6; p = 0.61; Mann-Whitney U-test; Fig. 10A). Next, a comparison of the survival fraction of new spines formed in the two groups within 4 days of SW training was performed, also not detecting a difference (survival fraction after 4 days: CTR, 0.22 ± 0.07, n = 13; SOD, 0.26 ± 0.09, n = 13; p = 0.92; Mann-Whitney U-test; Fig. 10B; survival fraction after 10 days: CTR, 0.09 ± 0.04, n = 7; SOD, 0.20 ± 0.09, n = 12; p = 0.97; Mann-Whitney U-test; Fig. 10B; survival fraction after 40 days: CTR, 0.03 ± 0.03, n = 4; SOD, 0.13 ± 0.07, n = 9; p = 0.71; Mann-Whitney U-test; Fig. 10B). In addition, mice were subsequently imaged after 2 days of CW training, there were still no differences in the survival fraction of newly formed spines after 2-day CW training between the SOD mice and the CTR mice (survival fraction after 8 days: CTR, 0.08 ± 0.08, n = 6; SOD, 0.12 ± 0.09, n = 11; p = 0.96; Mann-Whitney U-test; Fig. 10C; survival fraction after 38 days: CTR, 0.00 ± 0.00, n = 3; SOD, 0.04 ± 0.04, n = 8; p = 0.78; Mann-Whitney U-test; Fig. 10C); and also there were no differences in the survival fraction of new spines formed after 4-day CW training between the two groups (survival fraction after 6 days: CTR, 0.15 ± 0.05, n = 7; SOD, 0.20 ± 0.09, n = 12; p = 0.71; Mann-Whitney U-test; Fig. 10D; survival fraction after 36 days: CTR, 0.13 ± 0.06, n = 4; SOD, 0.06 ± 0.04, n = 9; p = 0.26; Mann-Whitney U-test; Fig. 10D).

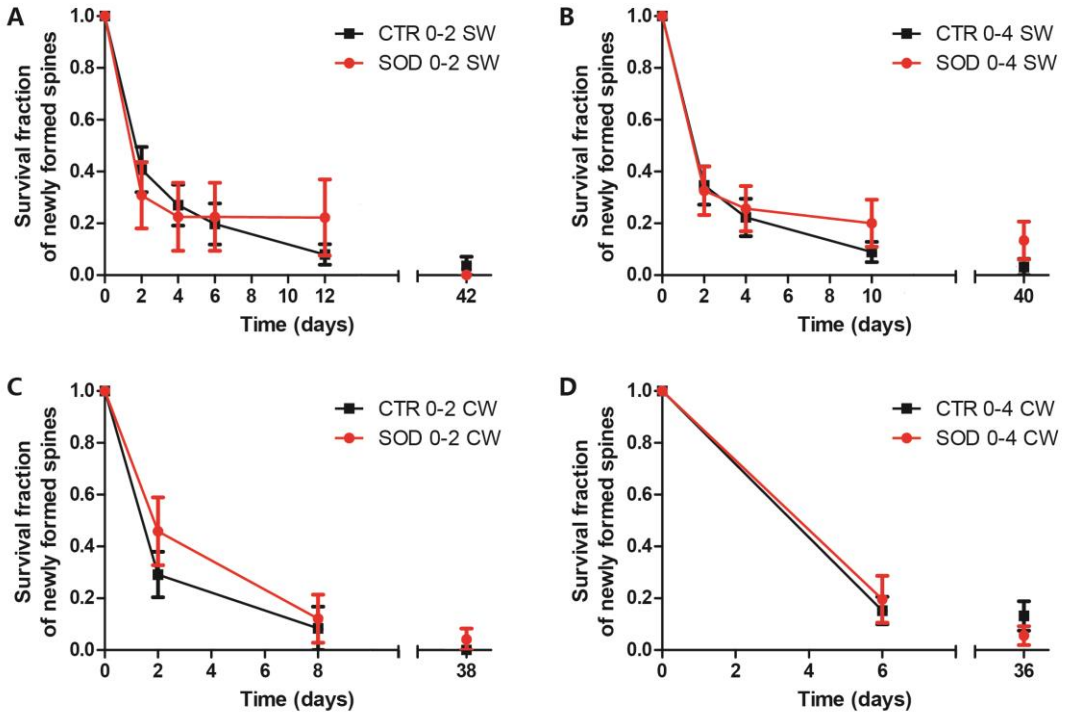

Figure 10. Survival fraction of newly formed spines after motor skill training.
(A) No differences were observed in the survival fraction of newly formed spines after 2 days of SW learning between the SOD mice and the CTR mice. (B) There was also no difference in the survival rate of newly formed spines after 4-day SW training between the two groups of mice. (C-D) Survival fraction of new spines formed after 2 days of CW training (C) as well as 4-day CW training (D) have also been compared respectively, there were still no differences between the SOD mice and the CTR mice. Data are indicated as mean ± s.e.m. Statistical significance is determined by $p < 0.05$.

In addition, correlations between the rate of spine formation and behavioral performance after SW learning, and between the rate of spine elimination and behavioral performance after SW learning as well as between the rate of spine turnover and behavioral performance after SW learning were then investigated separately for both groups. The formation rate, the elimination rate and the turnover rate of spines did not correlate with motor performance after SW learning (formation rate: CTR, Pearson's correlation coefficient $r = 0.07$, $p = 0.85$; SOD, $r = 0.20$, $p = 0.52$; elimination rate: CTR, $r = -0.39$, $p = 0.24$; SOD, $r = -0.22$, $p = 0.46$; turnover rate: CTR, $r = -0.28$, $p = 0.40$; SOD, $r = -0.02$, $p = 0.96$; Fig. 11A-F).

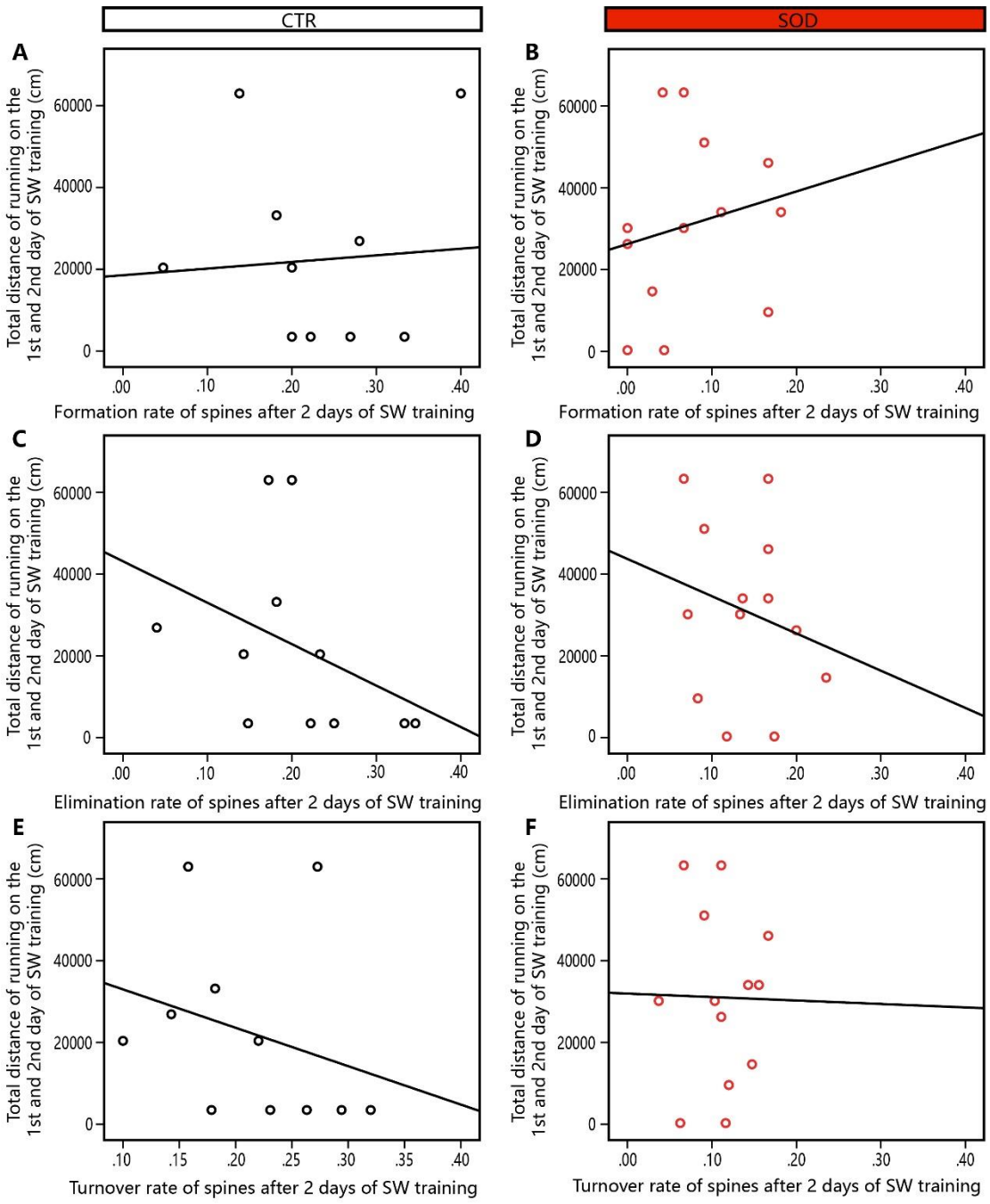

Figure 11. Spine formation rate does not correlate with behavioral performance after SW learning.

(A-B) The formation rate of spines does not correlate with wheel running distance in SW training for both CTR mice and SOD mice. (C-D) The elimination rate of spines was not correlated with running performance during SW training for both groups. (E-F) The turnover rate of spines was also not correlated with the motor performance during SW training for the CTR group and the SOD group. Correlations are indicated by Pearson's correlation coefficient (r). Statistical significance is determined by $p < 0.05$.

5. Discussion

In my thesis, the primary question of whether dendritic spines, the structural correlates of postsynapses, of LVPNs are affected during the presymptomatic phase in hSOD^{G93Adl} mice have been addressed. To this end, I performed chronic *in vivo* two-photon imaging in awake, behaving mice and monitored the same dendritic stretches and their spines over short and long periods and in association with a motor skill learning paradigm.

5.1 Behavioral experiments and the relevant potential pathophysiological mechanisms in SOD transgenic mouse model

As structural plasticity in particular in sensorimotor areas hinges on overall locomotion, I, first of all, assessed the locomotion and the exploratory behavior of CTR and SOD mice by performing an open field test. This test revealed that SOD mice were significantly less exploratory, with a lower average velocity and lower total distance traveled. The SOD mice also showed fewer entries into the center area of the arena and an increased total freezing time. The open field test alone is not suitable to evaluate functional motor deficits, which may interfere with exploratory behavior. However, since both the SOD score and the motor performance during the subsequent wheel skill learning paradigm were not altered, there is no indication of a motor deficit.

Earlier reports have unraveled subtle motor deficits in this hSOD1^{G93Adl} mouse model during the pre-symptomatic period. Tucci et al. used a motor function test of the Mouse Reaching and Grasping (MoRaG) performance scale to evaluate the reaching and grasping motor ability of mice, suggesting that this mouse model may be characterized by forelimb deficits and unbalanced posture from about 10 weeks of age[289]. Mandillo et al. designed a very detailed system in which a wheel device was placed in the home-cage of the mice to measure voluntary running activity. A total of three weeks of running activity were examined from 12 weeks to 14 weeks old, with a slight decrease in the distance at 12 weeks of age detected in the first hour of the dark phase[290]. Acevedo-Arozena A et al. employed a more commonly used method to measure motor

impairments, which is the accelerating rotarod test, and detected a decline in motor function in the male hSOD1^{G93Adl} mice only at 27 weeks of age. In addition, they detected deterioration of grip strength in the female hSOD1^{G93Adl} mice at 24 weeks of age as well as in the male hSOD1^{G93Adl} mice at 28 weeks of age, as well as the occurrence of startle-response deficits had been found in both hSOD1^{G93Adl} females and males at 22 weeks of age[262]. These motor function deficits observed at the presymptomatic stage in previous studies do not contradict my experiments, as the method of motor examination, the duration of the test, and the amount of the test are different, all of which could potentially affect the detection of subtle motor deficits. In addition, the present experiment showed a decreased exploratory behavior at the presymptomatic stage in SOD mice, suggesting that impairment of cortices other than the motor cortex may also occur in ALS[291-293].

The underlying mechanisms of the observed decreased exploratory behavior in the hSOD1^{G93Adl} transgenic mouse could be an impairment in the corticostriatal pathway [294]. Geracitano et al. found that the high-copy hSOD1^{G93A} showed a severe active avoidance learning deficit without detection of locomotor deficiency at 15 weeks of age, therefore, they proposed that these deficits were not due to hypokinesia, but rather to a defect in cognitive ability that arose from the functional impairments in the corticostriatal pathway. Indeed, they found that repetitive stimulation of the corticostriatal in-direct pathway in hSOD1^{G93A} mice did not produce an LTD phenomenon similar to those in control mice. Because no neuropathological alterations of the striatum were observed even in the final stages of ALS, the functional changes in this corticostriatal indirect pathway are postulated to be due to a decline of D_2 receptors (D2R) on the striatal medium spiny neurons (iMSNs)[294]. This hypothesis has also been confirmed by a study in ALS patients, Vogel and co-workers found that the expression of the D2R on iMSNs was downregulated in the sporadic ALS patients, besides, they further proposed that this may be due to the excessive neurotransmission of excitatory glutamate in the corticostriatal pathway[295,296]. Dorsal striatum plays a critical role in the planning and selection of movements especially under conflicts[297-299], with activation of the direct pathway facilitating desired movements execution, and

activation of the in-direct pathway inhibiting unwanted movements execution[300-304]. Therefore, the decreased exploratory behavior in this open field test of the hSOD1^{G93Adl} transgenic mouse is plausible due to a modification in the indirect corticostriatal pathway.

Another hypothesis has been proposed by Quarta et al. Similarly, they observed a reduction in visits to the center of the open arena and a reduction in the proportion of time spent in the central area, both of which preceded and dissociated from the detection of abnormalities in motor activity in the high-copy hSOD1^{G93A} mouse line at P56. In their further study, they suggested that loss of PV-positive interneurons in the dorsal hippocampus could partly account for the augmented anxiety-like behaviors in the open arena, which might be explained by the possibility that reduced GABAergic transmission would enhance the susceptibility to glutamate-mediated excitotoxicity in ALS [184,305], in turn, influence hippocampal functionality. In addition, the correlation of the anxiety-like behaviors in the OFT with the function of the hippocampus has also been shown by other studies[306-309], and the morphofunctional abnormalities of the hippocampus in ALS have been described before as well[182-185]. Therefore, the anxiety-like behaviors in the open arena exhibited in this 5- to 6-month-old pre-symptomatic hSOD1^{G93Adl} mouse line may also be due to hippocampal abnormalities.

Nevertheless, it is worth noting that the neural circuity for the fear or anxiety pathway is still not well understood and may involve not only the dorsal striatum, and the hippocampus, but also the amygdala, prefrontal cortex (PFC), hypothalamus, and the periaqueductal gray[281,310-313]. Therefore, further exploration is needed in this hSOD1^{G93Adl} mouse model as to where the culprit lies.

However, there is no evidence that the open field test affects spine dynamics in M1, therefore, this task has been adopted to facilitate the adaptation of the mice to the environment of the following voluntary wheel running test.

5.2 Dendritic spine pathology of LVPNs in presymptomatic hSOD^{G93Adl} mice in vivo

5.2.1 Spine alterations under baseline conditions

In this study, I tracked alterations in spine plasticity and changes in spine density in M1 in awake, behaving SOD transgenic mice. At least one segment of the apical dendrite of each mouse was selected to be tracked, and high-resolution image stacks were collected for each region of interest. In the presymptomatic period, the SOD mice, although not detected to have motor symptoms, have been detected markedly reduced spine density in comparison to the CTR mice, in agreement with previous results of in vitro studies of the presymptomatic hSOD1^{G93A} high-copy mouse models[5,170,314,315].
Previous in vitro studies have also employed mouse models of ALS to investigate dendritic spine pathology. Jara et al. retrogradely transduced UMNs by injection of adeno-associated virus serotype 2-2 (AAV2-2) eGFP into the corticospinal tract (CST) in the SOD^{G93A} mouse model. They successfully revealed the full cytoarchitecture of UMNs and found that apical dendritic spines in layer II/III are already reduced in number at P60 in SOD^{G93A} mice[316]. In the same mouse model, Fogarty et al. used a neurobiotin-filling method to visualize LVPNs, which to a large part are UMNs. Using confocal microscopy, they observed a regression of apical dendrites as early as P28 and apical dendritic spine density was decreased at P21[170]. With a Golgi-Cox impregnation method, this research group again found the dendritic arbors of LVPNs to be progressively reduced and apical and basal spines were significantly lost already at P28-35 in the SOD^{G93A} mouse model[314]. However, in contrast, they observed an increase of spine density at P26-35 in another different mouse model, TDP-43^{Q331K}. They proposed that this difference may be due to different rates of disease progression between the SOD^{G93A} and TDP-43^{Q331K} transgenic mouse model[171]. The TDP-43^{Q331K} mutant mice have a much slower progression rate and motor neurons in the lumbar spinal cord and cortex did not die until they were 24 months old[317]. Indeed, a relatively aggressive

mouse model, TDP-43^{A315T}, whose symptomatic stage is at P90, displays dendritic spine loss of LVPNs at P60[315].

The notion that different time courses and different capacities to cope with neurodegenerative processes may be associated with the occurrence of pathological features has also been raised by Pambo-Pambo et al. and evidenced by some studies investigating LMNs[139]. Pambo-Pambo et al. assessed the electrophysiological characteristics of LMN at P6-P10 in two transgenic mouse models: the SOD1^{G85R} line and the SOD1^{G93Adl} line, with different lifespans of 8 months and 7 months, respectively. They found that the more rapidly progressing SOD1^{G93Adl} mice exhibited hyperexcitability of motor neurons, manifested by an increase in frequency upon injected current stimulation, and by a more depolarized resting membrane potential (RMP). Whereas no such differences were detected between the SOD1^{G85R} mice and the wildtype (WT) control group[139]. With regard to LMN morphology, it has been found that LMNs in SOD1^{G85R} mice began to exhibit excessive elongation and branching at P3-P4[318,319]; while a reduction in dendritic morphology has been found at embryonic stages (E17.5) in the SOD1^{G93A} transgenic mice[47].

Along with the possible impacts of different rates of disease progression as well as the developmental impacts, an alternative hypothesis has been raised by Saba and colleagues. They proposed that neuronal hyperactivity may initially increase the formation of dendritic arbors, however, prolonged glutamate release may be detrimental and then inhibit the dendritic arborization. Evidence is provided by the SOD^{G93A} mice, which show longer and more ramified basal dendrites in LVPNs at 1 month of age[137], but exhibit a reduction in basal dendrite lengths (with comparable apical dendrite lengths) and synaptic plasticity defects assessed by the quantification of autophospho-alphaCaMKII (alphaCa^{2+}/calmodulin-dependent kinase) in the triton insoluble fraction at threonine-286 at 3 months of age[320].

Although degeneration of UMNs has been identified in brain samples from ALS patients over the last decades and is considered a characteristic manifestation of ALS disease[148,166,321-325], only in the last few years pathological changes of apical dendrites of UMNs have been identified for the first time in post-mortem brain tissues from ALS

patients[326]. The authors found that the apical dendrites of Betz cells in M1 (UMNs in humans) showed reduced post-synaptic density in sALS cases, and the synaptic markers, such as synaptophysin and PSD-95 were markedly reduced both in sALS patients and in fALS cases[326]. The findings on UMNs challenge the prevailing "dying-back hypothesis" and arouses attention to the UMNs dysfunction and its contribution to ALS[326]. Unfortunately, investigating spines in fixed-tissues in vitro only provides a static view. It precludes the monitoring of the dynamics of individual spines, which is a relevant characteristic of spine function[327]. Therefore, the present study adopted in vivo methods to explore how dendritic spines change under baseline conditions and in response to environmental demands, further confirming a decreased spine density already at the presymptomatic stage in SOD mice.

Moreover, in vivo methods allowed to chronically follow the spine dynamics. Results showed that the spine turnover rate was significantly lower in SOD mice compared to the CTR group not only over a two-day interval but also already during short-term intervals of 50-minutes, which may represent a difference in the capacity of the spine plasticity in M1 between the two groups of mice.

I also found a significantly higher fraction of spines in the SOD group still being present after 4 days compared to the CTR group, which means the fraction of spines with lifetimes less than 4 days in the SOD group was smaller than that in the CTR group, considering that the fraction of spines with lifetimes less than 4 days could be used as a measure of the stability of spines[216], therefore, spines in the SOD group are more stable in comparison to the CTR group.

These results indicate that under baseline conditions, structural plasticity is massively reduced in SOD mice, as not only are there fewer spines present but there are also way fewer spines gained and lost.

5.2.2 Experience-dependent spine modifications

In this experiment, I further explored whether additions and subtractions of spines would be influenced by the motor learning experience. To explore this question, mice

were imaged three times at the baseline level followed by voluntarily allowing mice to learn to run on a standard wheel and a complex wheel. The mice started to run in a wheel with regular rungs, which is the standard wheel. The running performance of mice gradually increased and finally reached a certain plateau. After 4 days of training on the SW, the mice were then trained on a wheel that had rungs removed and rungs loosened thereby making it more challenging for mice to maneuver it (complex wheel). As the rungs on the wheel became less regularly distributed, the mice needed to constantly adjust their stride length in order to adapt to running on this CW. Results showed that the mice complied very well and learned to run in the wheel voluntarily, except for one mouse that was excluded from the experimental cohort because it was reluctant to learn. My data shows that SOD mice during the presymptomatic phase perform just as well as CTR mice during the motor skill learning paradigm.

When investigating the impact of this motor skill learning paradigm on the structural plasticity of spines on the apical dendrites of LVPNs, I found that the spine formation rate increased in the CTR group after 2 days of the SW learning task, however, for the SOD mice, no significant difference was detected in spine formation rates compared to baseline levels. The design of our custom wheel differs from the standard wheel used in previous publications as it has a wider spacing between the rungs, and is heavier, thus more difficult to maneuver. We thus feel that already the standard version of our wheel represents a novel motor skill learning task[328]. The observed increase in spine formation in the CTR group is in agreement with a previous publication [221], in which a motor skill learning task (accelerated rotarod task) was also rapidly inducing a substantial increase in spine formation while the effect on the turnover rate and elimination rate was limited[221]. In addition, results showed that SOD mice did not exhibit a significantly increased spine formation rate after learning as in the CTR group, suggesting that the SOD mice lack the capacity to form spines rapidly within two days. Unexpectedly, chronic time-lapse imaging results showed that the CW learning paradigm did not cause significant changes in the dendritic spine density and formation rate compared to baseline levels. One possible reason for this is presumably due to the insufficient complexity and intensity of motor learning. The SW and the CW motor task

are quite similar[288], suggesting that CW learning is only a slightly more complex process compared to SW learning, rather than a novel type of learning.

However, this experiment provides important insight and experience for related explorations in the ALS transgenic mouse model, and further studies could increase the duration and complexity of motor learning appropriately to give mice a longer time to learn a more complex movement; alternatively, to increase the sample size to see if significant changes can be detected.

In addition, under baseline conditions, spines were more stable in the SOD group compared to the CTR group, as shown by a higher percentage of spines that survived for 4 days. However, the survival rate of new spines formed during 2-day SW learning or 4-day SW learning did not show a difference between the two groups, representing that the newly formed spines in SOD mice were eliminated for the same proportion as the CTR group. Such results suggest that SOD mice might have adapted to a more reduced level of structural plasticity over the prolonged disease course, in particular during the presymptomatic phase, so that their spines are in general less prone to change, in contrast, no such compensatory adaptation occurs for learning-dependent spine modifications.

Furthermore, the behavioral performance after SW learning did not correlate with the degree of structural plasticity both in CTR and SOD mice, which are consistent with Yang et al., who also showed that the learning performance is not simply correlated with the percentage of newly formed spines, but with an experience-dependent spine modification process[221].

5.2.3 Short-term spine plasticity

Previous studies showed that most dendritic spines remain stable over long-term intervals, such as days or months[218,221]. Nonetheless, little is known regarding how rapidly spines can form in awake mice. I here show that spines can emerge and vanish over short periods of time, with a mean of ~5% in the CTR group and ~3% in the SOD group, indicating that a handful of spines were lost and gained at rapid rates within a

50-minute interval. Earlier studies showed that spines are highly plastic during the developmental period, in particular, filopodia-like structures can change rapidly within tens of minutes, although the rate of change is small[219]. Another previous study had shown that spines are remarkably stable in V1 in vivo at time scales of 1-2 hours under anesthesia, with a turnover rate of zero[329]. I here however observed turnover rates of ~5%, suggesting that anesthetics may have some effect on the spine dynamics in mice. Furthermore, a weak, but significant positive correlation was found between short-term spine plasticity and running performance during 50-minute intervals on the styrofoam ball in the CTR group, which was not observed before. Combined with the results under anesthesia[329], this experiment may demonstrate a potential effect of locomotor activity on spine plasticity in M1, implying that exercise may cause an increase in short-term spine changes of LVPNs. However, such correlations were not detected in the SOD group, which might represent a deficit in rapid spine modifications in the SOD mice. Nevertheless, the effect of exercise on spine dynamics in the SOD mice cannot be denied[49]. In fact, the impact of exercise on the progression of ALS disease is constantly a controversial topic. The notion that strenuous exercise to be a risk factor relies on epidemiological studies, such as those of heavy workers, and on the widely reported cases of ALS among well-known athletes[41,49]. Whereas some evidence suggests that mild to moderate exercise is an active treatment for ALS, which can help motor function recovery and slow down ALS disease progression[49]. This experiment took the approach of having mice moving freely on the styrofoam ball for about 2h every other day and did not observe a correlation between the locomotion and the spine plasticity of LVPNs in the SOD mice. Given the large heterogeneity of exercise methods reported in various studies, preventing the data from the direct comparison[41]. Whether other motor paradigms are more potent in stimulating structural plasticity and whether exercise would be beneficial for UMNs and the survival of SOD mice warrants further exploration through additional and more extensive laboratory studies in order to elucidate.

5.2.4 Molecular mechanisms mediating structural plasticity of spines

Various studies have previously investigated the molecular mechanisms mediating spine plasticity. Experience-dependent synaptic remodeling allows the synapses to encode novel information in order to adapt to the new environment [226]. Electrophysiological processes such as long-term potentiation (LTP) and long-term depression (LTD) can alter synaptic strength[212]. Therefore, LTP and LTD have been proposed as the primary mechanisms mediating activity-dependent synaptic plasticity in the mammalian brain[330]. During LTP, an influx of Ca^{2+} is induced by postsynaptic depolarization through the NMDAR channels, which are the relatively stable constituents of the PSD, subsequently triggers the accumulation of AMPARs, the polymerization of actin filaments, calcium/calmodulin-dependent protein kinase II (CaMKII) activation and the following plasticity-related proteins (PRPs) synthesis[330,331]. LTP can be induced by electrical stimulation or by exposure to chemical agents, such as Ca^{2+}, metabotropic glutamate receptor agonist aminocyclopentane-1S,3R-dicarboxylate, K^+ channel blocker, tetraethylammonium (TEA), arachidonic acid, and G-protein activator[332]. Furthermore, multiple experiments have demonstrated that in vivo experience also induces LTP or LTD phenomena at specific synapses and such modifications of synapses might serve essential functional roles[332-336]. However, simply putting an LTP mechanism here obviously oversimplifies the issue， as has been discussed in the review by Malenka et al., LTP and LTD might not be the only means that mediate the activity-dependent synaptic modification[330,337,338].

Martin et al. highlight another hypothesis, which is the synaptic plasticity and memory (SPM) hypothesis[339]. At the heart of this hypothesis is the idea that synaptic plasticity and changes in synaptic efficacy last longer than the duration of the events that trigger these changes[339]. This notion has been developed with two main factors in mind. One is the influence of the specific neural networks involved in synaptic plasticity, and the other is the presence of different neural activity that induces synaptic plasticity, such as

when there are alterations in neuromodulatory input[339].

Another plasticity-pathology continuum model emphasizes the importance of neurotransmitter receptor regulations in synaptic modifications. Based on the fact that this regulation is different in adult and young brains, the former being homeostatic and the latter being homeodynamic[340]. This model could provide a theoretical basis for a phenomenon observed in ALS, which is the downregulation of NMDAR subunits due to overactivation by excitatory glutamate in both *in vivo* and *in vitro* models[341].

5.2.5 Potential mechanisms implicated in ALS spinopathy

Molecular mechanisms governing spine plasticity with regard to ALS are undoubtedly quite complex[206]. As a dynamic structure, dendritic spines undergo changes in morphology and number to modulate the synaptic connections throughout life[6,342]. Experience-dependent spine modifications are not only prominent under healthy conditions but also when it comes to disease processes, such as in psychiatric and neurodegenerative disease[187,200,214,231]. Functional alterations originating from the excitatory postsynaptic changes have been identified as pivotal and contributing to the adaptation of motor cortical neuronal circuity during the long presymptomatic period in ALS cases[135].

By comparing the studies of spines in different ALS mouse models, it is hypothesized that one of the mechanisms leading to the reduction in spine density may be that the capacity to deal with excessive cellular stress in the early stages of the disease is progressively insufficient as the disease proceeds, giving rise to a reduction in spine density. Evidence was provided that the rapidly progressing hSOD1^{G93A} transgenic mice showed a loss of spines at 1 month of age[314]; whereas the slowly progressing TDP-43^{Q331K} mutant mice were not detected to have a reduction in spine density at 1 month old[171]. Another possible mechanism is that excessive glutamate may favor the growth of dendrites and dendritic spines in earlier stages of ALS but gradually becomes detrimental as the disease progresses[137]. The exact mechanism underlying the decrease in spine density remains for further exploration.

In parallel, I also observed reduced spine dynamics and increased spine stability in the SOD mice, as indicated by a decreased formation and elimination rate of spines, and a higher survival fraction of pre-existing spines. The process of spine formation and elimination is complicated and remains largely unknown[212,330]. Polymenidou et al. revealed that more than 600 mRNAs showed abnormal levels by genomic analysis in the TDP-43 ALS mouse model, especially those with long pre-mRNAs and encoding proteins associated with synaptic activity[118]. Through a real-time study of the substructures of dendritic spines at single synapse resolution, Bosch et al. found that the formation, stabilization, and maintenance of a spine involved many molecules and proteins, including neurotransmitter receptors, signaling complexes, scaffolding proteins, actin filaments, and actin-regulatory proteins[343], as well as the finely coordinated cooperation of a number of cellular organelles spatially and temporally[214]. As is readily understood, in different diseases, given that the pathogenic mechanisms are different, the impact on spine substructures may vary, and correspondingly, the impact on the formation and maintenance of spines may also vary. In ALS, structural plasticity seems to depend on proteasomal function[344], which is frequently impaired in ALS. While proteasomal dysfunction is established for LMN, it remains open whether UMNs in rodents and humans are also compromised, in particular already early postnatally, when spine density reduction is observed. Gorrie et al. studied an ALS/dementia-linked UBQLN2^{P497H} transgenic mouse model and observed reduced spine density and synaptic dysfunction in the dentate gyrus molecular layer. They proposed that the abnormal ubiquitin-proteasome system (UPS) caused by the mutated UBQLN2 gene, which results in off-regulation of the turnover of synaptic proteins, is responsible for the dendritic spinopathy[345]. Dysfunction of the proteasome-mediated protein degradation has also been involved as a pathological feature mediated by the mutant SOD1 gene[74], and therefore, may have a consequential effect on spine plasticity in SOD1 transgenic mice as well.

Spanloni et al. studied M1 in the high-copy hSOD^{G93A} mice at the presymptomatic stage and detected a decreased protein expression level of NMDAR subunit NR2A (but normal expression levels of AMPAR subunits), and a significant decrease of

autophosphorylation at threonine-286 of the alphaCaMKII protein, which is a critical signaling molecule and a primary component of the PSD, pointing out that these molecular abnormalities may cause a decrease in the susceptibility of the motor neurons to LTP, which in turn leads to the impairment of spine formation and a reduction in the number of apical dendritic spines[320].

Pradhan and co-workers proposed that brain-derived neurotrophic factor (BDNF) not only does not produce neuroprotective and trophic effects in ALS, but on the contrary, may even enhance the glutamatergic activity, which in turn enhances excitotoxic insults, and accelerates the glutamate-induced neuronal cell death[346-352].

In a study of cerebral ischemia, Hasbani et al. found that the postsynaptic structures became vulnerable after the use of glutamate receptor agonists, and dendritic swelling and spine loss occurred[327]. Glutamate excitotoxicity, which is caused by the excessive glutamate in the synaptic cleft, is an important pathological hallmark of ALS, besides, excessive glutamate may contribute to loss of spines in ALS transgenic mice was also proposed by Saba et al[137]. It is therefore plausible that similar activation of excitatory amino acid pathways is also involved in the pathological alterations of spines in ALS and resulting in spine loss.

During the process of synaptogenesis, it has been found by Li et al. that mitochondria are required to move toward the dendritic protrusions and to supply ATP regionally, and a reduction of dendritic mitochondria content will result in a decline in the plasticity and number of spines[353,354]. Since dysfunctions of mitochondria have been found in numerous studies in the ALS transgenic mouse tissues as well as in post-mortem tissues, it is likely that these abnormalities may also be implicated in the pathogenesis of spinopathy in ALS[355-357].

In addition, along with the proteasome, the endoplasmic reticulum is also involved in the regulation of protein homeostasis because of its functions such as processing, modification, and transport of proteins[123]. Therefore, due to their important role in maintaining the homeostasis of intracellular synaptic proteins metabolism, altered functions of these organelles may also be implicated in the pathological changes of ALS spinopathy.

Another possibility has been proposed by Rochefort and co-workers, based on the observations from Chen JL et al, Gambino F et al., and van Versendaal D et al.[237-239]. They pointed out that not only the pruning of dendritic spines is triggered by experience, but the development of interneurons is also activity-dependent[254,358-361]. In ALS, not only are there abnormalities in the number and property of interneurons but there is also a disturbance of the intracortical interneuron circuitry. Furthermore, it has been found that the patients with an intact intracortical interneuronal circuitry had slower disease progression[362]. Therefore, it is plausible the abnormal interneuronal capacity may also have an impact on spine dynamics.

However, whether the above hypotheses are indeed implicated in the pathogenesis of spine pathology in ALS (Fig. 12), in which sequence they evolve, which one serves the most critical role, and which ones can be used as targets for therapeutic interventions also need to be confirmed by real-time studies at the synapse level[343].

It is worth noting that one could not conclude that spines lack the capability to remodel in this transgenic mouse model, since this experiment did not investigate another way of modifying the spine efficacy to process the input information, which is through enlargement and shrinkage of the spine volume. In fact, in a companion study, by performing stimulated emission depletion (STED) imaging, we could show that spine heads in hSOD1^{G93Adl} mice were on average larger than in CTR mice, suggesting some compensatory effect[222]. As larger spine heads are also believed to possess more powerful synapses this effect could potentially partially explain the increased excitability of UMNs[135] thereby establishing a link between structural and functional alterations.

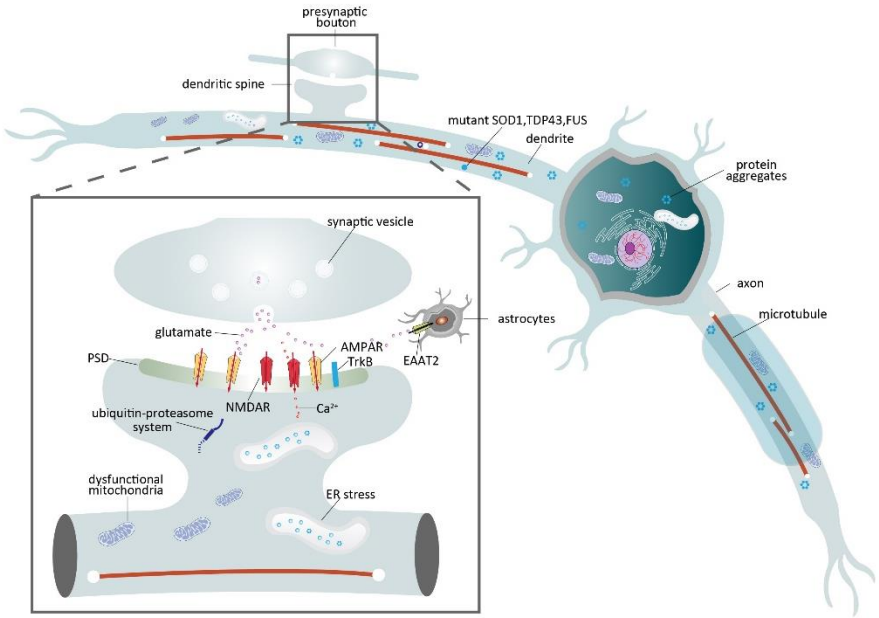

Figure 12. Pathophysiological mechanisms potentially involved in the spinopathy in ALS.

BDNF-TrkB pathway fails to produce neuroprotective and trophic effects in ALS[352]. The abnormal ubiquitin-proteasome system leads to impairment of the turnover of synaptic plasticity-related proteins[344]. Down-regulated expression of the signaling molecules, such as NMDAR subunit NR2A, may cause a decrease in the susceptibility of motor neurons to LTP, which may impair the formation of a spine[320]. Excessive glutamate may cause the postsynaptic structures to be vulnerable[327]. Reduction of dendritic mitochondria and mitochondria dysfunction may contribute to the reduction of spines[355-357]. Endoplasmic reticulum stress and dysfunction in maintaining the homeostasis of synaptic proteins may also be involved in the reduction of dendritic spine density and plasticity of ALS[123].

6. Conclusion

In my thesis, I performed structural imaging to assess individual dendritic spines of UMNs in pre-symptomatic hSOD1^{G93Adl} mice to assess their density and dynamics under baseline conditions and their short-term and experience-dependent plasticity. I found that there was a decrease in spine density, a decrease in spine dynamics, while the spine stability of the remaining spines increased. Furthermore, when exposed to learning a novel motor skill, the rapid increase in spine formation rate that could be observed in the CTR mice within 2 days was absent in SOD mice, confirming that the capacity for rapid spine formation in the SOD mice was compromised.

Taken together, my data demonstrates a pronounced impact on dendritic spines of UMNs in a mouse model of ALS, evident already during the presymptomatic phase, further corroborating the notion of ALS being a synaptopathy.

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Abbreviations

AAV2-2	adeno-associated virus serotype 2-2
AD	Alzheimer's disease
AIS	axon initial segment
alphaCaMKII	alphaCa^{2+}/calmodulin-dependent kinase
ALS	amyotrophic lateral sclerosis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AP	anterior-posterior
APs	action potentials
ARRIVE	Animal Research: Reporting of in Vivo Experiments
ASOs	antisense oligonucleotides
ATF3	transcription factor 3
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
C9ORF72	chromosome 9 open reading frame 72
CAM	cell adhesion molecules
CaMKII	calcium/calmodulin-dependent protein kinase II
CHCHD10	coiledcoil-helix-coiledcoil-helix domain-containing 10
CHOP	C/EBP homologous protein
CSMN	cortical spinal motor neuron
CST	corticospinal tract
CTR	control
D2R	dopamine D2 receptor
DG	dentate gyrus
DPRs	dipeptide repeat proteins
EAAT2	excitatory amino acid transporters
EDL	extensor digitorum longus
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
fALS	familial amyotrophic lateral sclerosis
FDA	U.S. Food and Drug Administration
FF	fast fatigable motor neurons
FIP-2	14.7-kDa interacting protein
FTD	frontotemporal dementia
FUS	fused in sarcoma
GABA	gamma-aminobutyric acid
GADD153	growth arrest- and DNA damage-inducible gene 153
GLT1	glutamate transporter 1
GOF	gain-of-function
GRN	granulin
HD	Huntington's disease

hnRNP	heterogeneous nuclear ribonucleoprotein
Hsp25	heat shock protein 25
Hsp27	heat shock protein 27
iMSNs	medium spiny neurons
IT-type neurons	intratelencephalic neurons
LMN	lower motor neuron
LOF	loss-of-function
LTD	long-term depression
LTP	long-term potentiation
LVPNs	layer V pyramidal neurons
M1	primary motor cortex
MATR3	matrin 3
MD	monocular deprivation
ML	medio-lateral
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin-dependent autophagic pathway
NF	neurofilament
NMDAR	N-Methyl-D-aspartic acid receptor
NMJ	neuromuscular junction
OD	ocular dominance
OFT	open field test
OPTN	optineurin gene
PD	Parkinson’s disease
PFC	prefrontal cortex
PRPs	plasticity-related proteins
PSD	postsynaptic density
PSD-95	postsynaptic density protein-95
PT-type neurons	pyramidal tract-type neurons
PV	parvalbumin-immunoreactive interneurons
RMP	resting membrane potential
sALS	sporadic amyotrophic lateral sclerosis
SICI	short-interval intracortical inhibition
sIPSCs	spontaneous inhibitory postsynaptic currents
SOD1	Cu/Zn superoxide dismutase 1
SPM	synaptic plasticity and memory
STED	stimulated emission depletion
TARDBP	transactive response DNA-binding protein
TDP-43	TAR DNA binding protein 43
TEA	Tetraethylammonium
TEC	transentorhinal cortex
TOR	turnover rate
TrkB	Tropomyosin receptor kinase B
UBQLN2	ubiquilin 2
UMN	upper motor neuron

UPR	unfolded protein response
UPS	ubiquitin-proteasome system
VCP	valosin-containing protein
WT	Wildtype

List of tables

List of figures

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Due to my limited academic level, the doctoral thesis I wrote inevitably has shortcomings, and I sincerely welcome all professors and peers to criticize and correct.

Affidavit

Affidavit

Siyuan Li

_____

Surname, first name

I hereby declare, that the submitted thesis entitled:

Comprehensive assessment of dendritic spine dynamics in the primary motor cortex of the SOD1^{G93A} transgenic mouse model of ALS

is my own work. I have only used the sources indicated and have not made unauthorized use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.
I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 2/9/2021 Siyuan Li

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place, date Signature doctoral candidate