NEUROMUSCULAR JUNCTION STRUCTURAL CHARACTARISTICS IN FAMILIAL ALS

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ABSTRACT

INTRODUCTION: In the spinal cord and cortex of the motor nerves, amyotrophic lateral sclerosis (ALS) is a deadly neurodegenerative disease. Because copper-zinc superoxide dismutase-1 (SOD1) mutations account for 10% to 20% of familial ALS cases, most instances of the disease are not hereditary.

OBJECTIVE: Studying neuromuscular junctions in various age groups to see how they vary in structure.

METHODS: Women's G93ASOD1 transgenic mice and WT littermate partners were generated from male hemizygous carriers and female B6SJL-Tg1Gur/1Gur hybrids. All of the animals were bought from Jackson Laboratory, Inc. Sterilized rodent chow and sterile food without particular pathogens was supplied for the animals, which were housed in an environment with regulated humidity and temperature.

RESULTS:

40, 60, 90, and 120-day-old mice have WT and SOD1 mutations. The gastrocnemius (GN) and Tibialis anterior (TA) of G93ASOD1 mice were constructed. Presynaptic terminus is shown by the neurofilament heavy polypeptide (tubulin, green). BTX (white) and synaptophysin (p38-1, red) identify the postsynaptic space and synaptic vesicles, respectively. WT and SOD1 did not deteriorate in 40-day-old mice, although a minor denaturation occurred in WT and SOD1 in 60-day-old animals.

Furthermore, the deterioration in mice after 90 days is only 60-70 percent significant.

CONCLUSION: NMJ innervation has emerged. 60 and 90-day disappearance, motor axon loss and severe structural damage to the NMJ. The presynaptic and postsynaptic structures have undergone structural alterations, and synaptic vesicles have been steadily decreasing.

Keywords: **Amyotrophic lateral sclerosis, neuromuscular junction, Synapse, SOD1**

1. INTRODUCTION

Motor neurons in the brain and spinal cord begin to degenerate, resulting in muscle atrophy, weakness, and paralysis. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative illness. A small percentage (around 10%) of ALS cases may be traced back to a family member. As many as 20% of familial ALS cases are caused by SOD1 gene mutations, while 5% of sporadic ALS cases are caused by SOD1 gene mutations [1–2]. The actual cause of ALS is still unknown, and no treatment has made a significant difference in the lives of patients. As the disease progresses, it affects all of the patient's voluntary muscles. Denervated atrophy, myopathy, muscular fibrosis, and inflammation are all common causes of muscle disease.

[6] Skeletal muscles make up almost 40% of the body's lean mass, therefore they need to have adequate systems for recycling damaged or old organelles as well as endurance to fulfill physical demands. Keeping muscle metabolism and mobility running smoothly relies on a steady flow of autophagic flux. Impairment of autophagic flow may lead to muscle atrophy and degeneration via inflammation, oxidative stress and aberrant mitochondrial breakdown [7–14]. Both nerve regeneration and NMJ reinnervation inside the target muscle are critical to functional motor recovery after peripheral nerve damage. Nerve-muscle junction (NMJ) is made up of three main structures: the nerve terminal, which contains acetylcholine (ACh) vesicles; the motor endplate covered in acetylcholine receptors (AChR); and 3 to 5 nonmyelinating terminal tSCs, or per synaptic, that encase the nerve terminal and the synaptic cleft. [15-17] The NMJ is not a static structure since it undergoes continual remodeling during the animal's life and after an injury. It is a dynamic structure. [16,17] Many studies have focused on nerve regeneration at the lesion site, but the mechanism of NMJ re-innervation following nerve damage is still a mystery. [18,19]. Axonal loss of the motor neuron axon results in the loss of the post-synaptic apparatus, which is the first pathological event in ALS [20]. [21] A variety of presymptomatic alterations, including sensitivity to environmental stressors like hypoxia, have been found in models of mSOD1 [21]. Several neuronal compartments, including the NMJ, have been shown to respond to environmental stimuli [21]. Furthermore, ALS degeneration is triggered and modulated in distinct compartments, with somal degeneration seldom extending to protection of distal axons or improved clinical outcomes in animal models of peripheral motor neuropathy, bax deletion and mSOD1G93A. Because of this, degradation of the distal components is a critical phase in ALS. However, we don't know exactly how things will alter. The post-synaptic alterations will assist establish the function of muscle or motor neuron in the causes of NMJ degeneration.

2. MATERIAL AND METHODS

All of the male hemizygous carriers (B6SJL-1Gur/J) and female B6SJL-F1 hybrids were bred to produce male and female G93ASOD1 transgenic mice and WT littermate mates. Controlled humidity and temperature, 12:12h light in a dark cycle, and sterile rodent food and sterile food without particular pathogenicity are supplied for the animals.

Muscle Harvest

Animals were sedated at 40, 60, 90, and 120 days of age, and a preposterous right leg was amputated from the dorsal foot to the knee. The Gastrocnemius (GS) and Tibialis anterior (TA) distal ligaments were severed after a recognized evidence. Proximally, the TA and GS

were meticulously dismantled in order to avoid injuring the nerve where it is embedded. In order to remove hair and other debris from the TA and GS muscles, they were first analyzed, then placed on the gum Tragacanth (GT) and immersed in 2-methylbutane for 10 to 15 seconds (cold by fluid nitrogen). Preserve strength in the lower 20s Celsius. Cervical separations were performed on animals while they were sedated. In all cases, the National Institute of Health and the Chinese Ministry of Science and Technology developed rules for the care and use of experimental animals, and those standards were strictly adhered to.

Following the manufacturer's instructions, RNA was extracted using Trizol and retrotranscribed using the Taqman reverse transcription kit (Servicebio; Cat.No: G3013). SYBR Green Master Mix (ABI Cat. No. Stepone plus) was used for qRT PCR, following the manufacturer's recommendations, as shown in the figure. GAPDH was used as a housekeeping gene to normalize the relative expression data. The following is a list of available primer sequences. Primers used in real-time PCR to amplify mRNA

Gene symbol	Forward primer	Reverse primer
YH1	TTCAAGTTTGGACCCACGGT	TTCTGAGCCTCGATTCGCTC
MYH2	TTTGCCAGTAAGGGTCTGTGAG	GCTCCGCCACAAAGACAGAT
MYH4	AAGCCTGCCTCCTTCTTCATC	CTTAGCATCCACCACAAACAC
MYH7	CCCAGAAACAAGTGAAGAGCCT	GTTCCACGATGGCGATGTTC
GAPDH	CCTCGTCCCGTAGACAAAATG	TGAGGTCAATGAAGGGGTCGT

For all preparations, ultrathin slices (10um) were cut using a microtome with a Diatom diamond knife (Leica Microsystem, Austria) (Diatomite. CH-2501 Biel, Switzerland) Glass slides (adhesion microscope slides) were used to fix serial transverse cryosections (10 M) in place. For 15 minutes, tissue was permeabilized with 0.3 percent Triton X-100 diluent and Tween (Sigma-Alorich) in PBS for 3x10 minutes (membrane antibodies do not require this step). Incorporated the dunky serum into the skin for an hour and a half (as protocol). Tissue was washed three times with PBS and incubated overnight at 4C with primary and secondary antibodies, respectively, at room temperature for one hour. The secondary antibodies were incubated for two hours at room temperature in the dark before being washed away. Tissue was counterstained with DAPI (4',6-Diamidino-2-Pheylindole) just after

the secondary antibody was removed. Synaptophysin-1 (P-38, 1:500, cat.no;101022,poly clonal rabbit antibody, 37079 Göttingen Germany); Alpha Tubulin (tubulin, 1:500, cat.no; 66031-1-ig,mouse monoclonal antibody, proteintech); -Bungarotoxin (BTX, 1:500, CF dye conjugates); goat anti-rabbit Alexa Fluor 488 (1:1000; Thermo Fisher; Cat. No: A-11034); or goat anti-mouse Alexa Fluor 594. (1:1000; Thermo Fisher; Cat. No: A-11037). DAPI Fluoromount-G was used to counterstain the nuclei (Southern Biotech). Fluorescence conofocal microscopy was used to examine the slides (Olympus FV1000). The experimental setup contains the laser power value, HV, gain, and offset parameters for each channel, which are calculated at the beginning of each imaging process (by measuring the background reactivity and saturation level of each channel) and stay constant during the imaging process. Each Z-stack generated a colocalization channel, which Olivia then measured based on the overlap. The ratio of positive cells to DAPI in five 20 magnification fluorescence areas showed the proportion of positive cells. Fluorescence pictures at five x 20 magnification were used to calculate the proportion of fibrosis. The studies were carried out three times, each time with six mice. The fluorescence quantification was analyzed using Image-Pro plus 5.1 software.

3. RESULTS

WT and SOD1 were found in 40-day-old mice, according to the data. The gastrocnemius (GN) and Tibialis anterior (TA) of G93ASOD1 mice were constructed. The amount of neuromuscular junctions (NMJ) in different assemblages may be seen in the image (below). Presynaptic terminus is shown by the neurofilament heavy polypeptide (tubulin, green). To identify synaptic vesicles and postsynaptic space, we utilize A-Bungarotoxins (BTX, white) and p38-1 (red). This shows that none of them has decomposed and is 90% complete, while in the course of 60 days, mice have degraded and are 90% completed For WT and SOD1, the denaturation was found to be 70-80%, which indicates that there has been some degradation, but in mice over 90 days, the degeneration was found to be 60-80%, which is not significant.

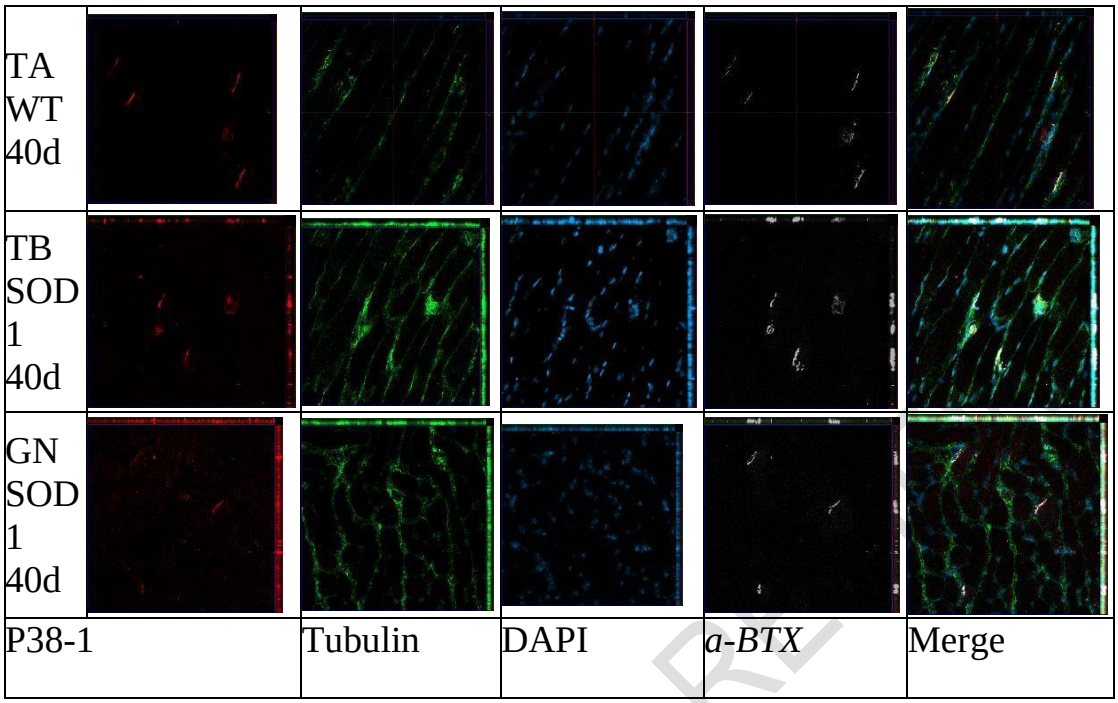
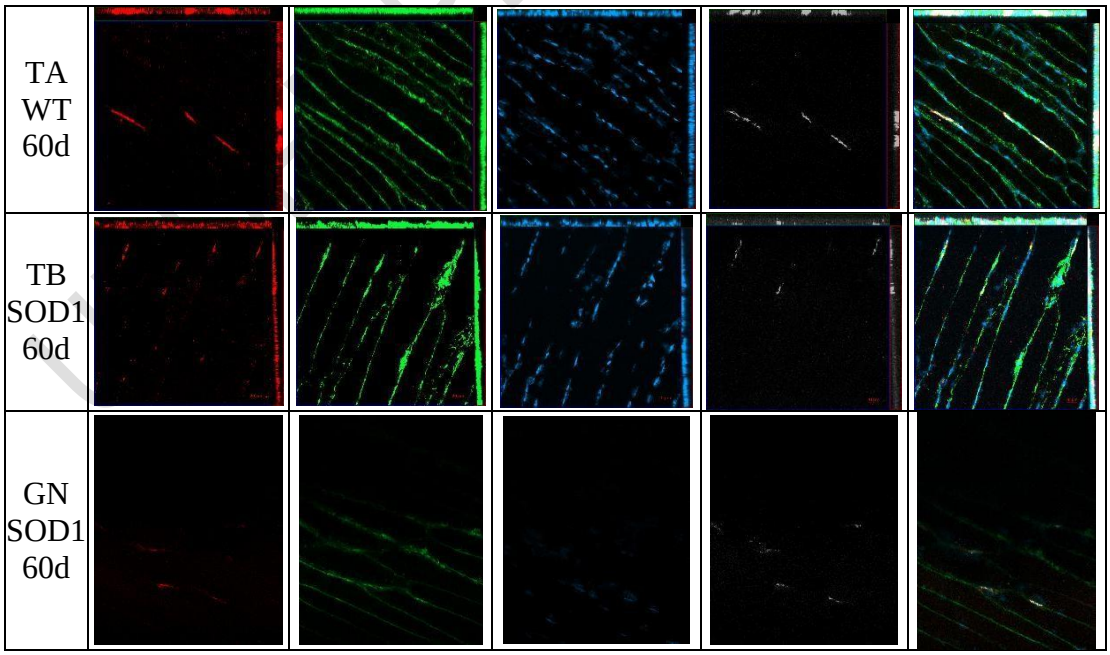


Fig.1. 40-day-old mice have the WT and SOD1 genes. The gastrocnemius (GN) and Tibialis anterior (TA) of G93ASOD1 mice were constructed. The number of neuromuscular junctions (NMJ) in distinct groups is shown in the image (above). Presynaptic terminus is shown by the neurofilament heavy polypeptide (tubulin, green). BTX (white) and synaptophysin (p38-1, red) identify the postsynaptic space and synaptic vesicles, respectively.



P38-1	Tubulin	DAPI	α -BTX	Merge

Fig.2. 60-day-old mice have the WT and SOD1 genes. The gastrocnemius (GN) and Tibialis anterior (TA) of G93ASOD1 mice were constructed. The number of neuromuscular junctions (NMJ) in distinct groups is shown in the image (above). Presynaptic terminus is shown by the neurofilament heavy polypeptide (tubulin, green). BTX (white) and synaptophysin (p38-1, red) identify the postsynaptic space and synaptic vesicles, respectively.

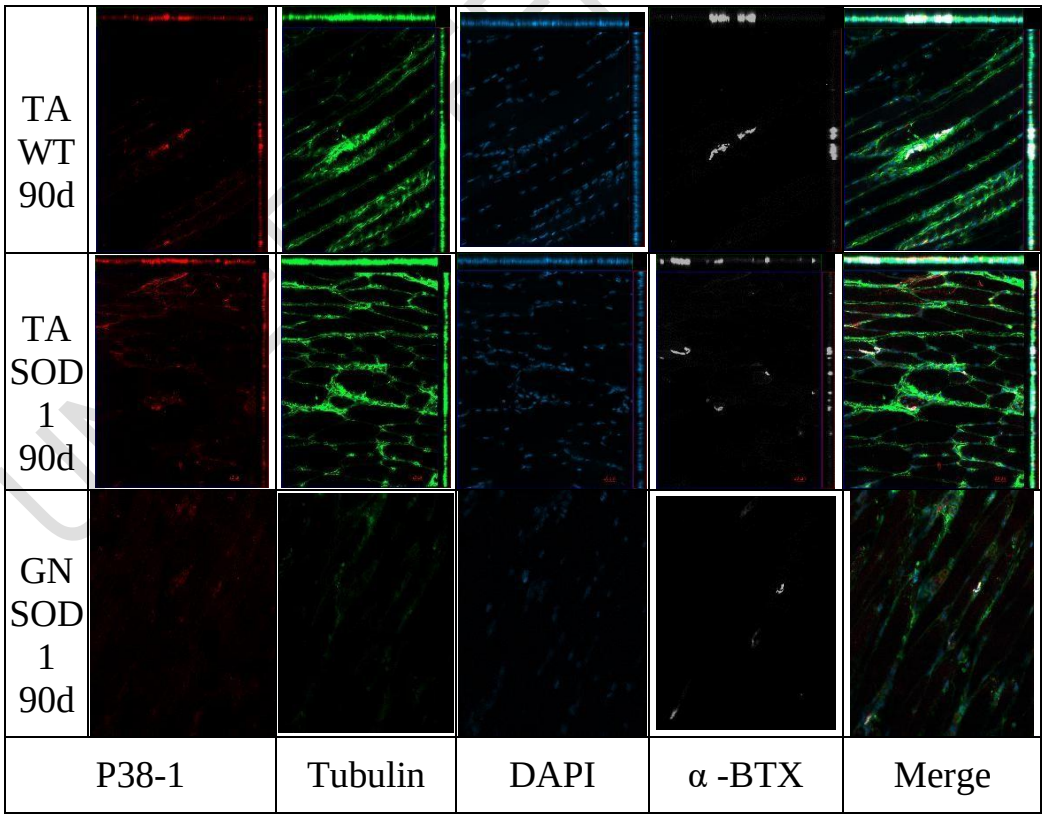


Fig.3. Mouse strains that are 90 days old have both WT and SOD1. The gastrocnemius (GN) and Tibialis anterior (TA) of G93ASOD1 mice were constructed. The number of neuromuscular junctions (NMJ) in distinct groups is shown in the image (above). Presynaptic terminus is shown by the neurofilament heavy polypeptide (tubulin, green). BTX (white) and synaptophysin (p38-1, red) identify the postsynaptic space and synaptic vesicles, respectively.

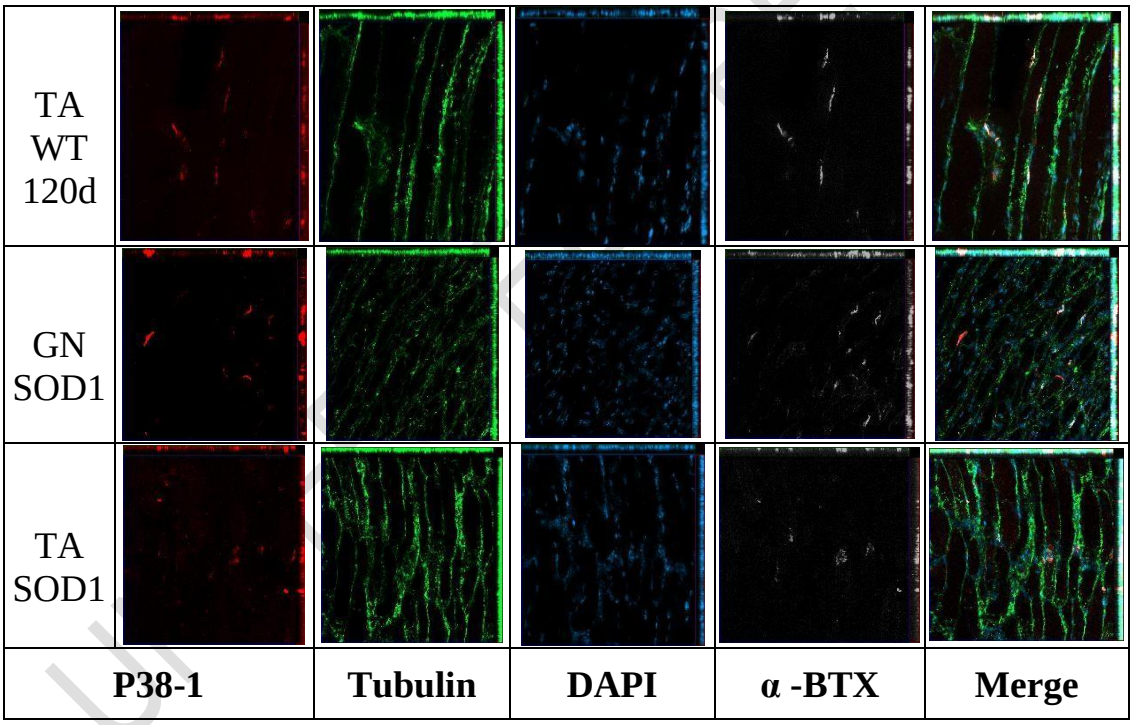


Fig.4. WT and SOD1 are found in mice that are 120 days old. The gastrocnemius (GN) and Tibialis anterior (TA) of G93ASOD1 mice were constructed. The number of neuromuscular junctions (NMJ) in distinct groups is shown in the image (above). Presynaptic terminus is shown by the neurofilament heavy polypeptide (tubulin, green). BTX (white) and synaptophysin (p38-1, red) identify the postsynaptic space and synaptic vesicles, respectively.

4. DISCUSSION

Researchers in this research found that by 40 days, NMJ innervation had disappeared, motor axons had been lost, and the NMJ was severely damaged structurally at 60 and 90 days, based on observations of the structural properties of the neuromuscular junction in WT and SOD1 mice. We found structural alterations in presynaptic and postsynaptic structures, as well as a reduction in synaptic vesicles, as a result of our research. To test whether these systemic alterations may be a therapeutic target, we looked at the underlying chemical mechanisms that cause them. One may distinguish between two types of muscle fibers in mammals' skeletal muscles: type 1 (mild jerk) and type 2 (strong pull) (quick jerk). There are three subtypes of type 2 strands, which may be further divided into type 2A (fast oxidative), type 2B (rapid glycolytic), and type 2X (medium glycolytic) [26, 27]. Undeveloped MyHCs are first expressed by regaining muscle, but soon mature into mature and fast MyHCs [28]. Early stages of myosin development do not need innervation, but the transition from rapid myosin to moderate myosin required the involvement of the slow nervous system [63]. In ALS mice, a rapid to moderate change in fiber type has already been accounted for [29,30]. However, re-innervations of healing muscle have demonstrated that the rapid to moderate change may be an aftereffect. Growing evidence suggests that SOD1 may have a role in aging-related sarcopenia and in the etiology and progression of neuromuscular illnesses, such as amyotrophic lateral sclerosis (ALS), in addition to motor neurons. Disassembly of the NMJ may or may not be a pathogenic event that happens as a result of the underlying abnormalities in motor neurons, and there is some debate regarding this. SOD1G93A mice (7) were used as a model to investigate this subject because they provide an appropriate model to separate the universal harmful effects of mutant SOD1G93A (10) from the tissue-specific effects. As a result, the animal model, which produces the toxic mutant protein in all tissues, was unable to determine whether cell type, either motor neurons or muscle fibers, may have initiated NMJ as a result of oxidative damage produced by the toxic action of SOD1. It is still debated whether motor neuron impairment in ALS should be considered a dying forward phenomenon, in which primary damages occur in motor neurons in the cortex

(i.e. through glutamate excitotoxicity or altered neuronal excitability) and then extend anterogradely to corticospinal projections, or if ALS should be considered a distal axonopathy, in which motor neuron d If you think about the intricacy of the ALS pathogenesis, it's plausible to assume that both ALS's forward and backward processes may take place at the same time and that skeletal muscle denervation is a significant step in ALS clinical symptoms start and pathogenesis, regardless of the progression mode. According to the idea that ALS is not a cell-specific illness, it is based on the finding that in addition to motor neurons, other cell types are destroyed and exhibit abnormal behavior. Furthermore, this has been shown in the last fifteen years by producing ALS-linked mutant proteins in tissue or cells[39]. In animal models, production of ALS-linked mutant proteins has been shown to cause an ALS phenotype, although whether or not this is sufficient is still up for dispute. Neuron-specific mutant SOD1 expression was not sufficient for disease development in early investigations on mice [40,41]. The production of chimerical mice supported this finding, demonstrating that mutant SOD1 in neurons was not harmful even in the absence of mutant SOD1 expression in non-neuronal cells [42,43]. A few years after this, it was shown that a little amount of mutated SOD1 in neurons was enough to cause an ALS phenotype in mice [34]. A possible explanation for these discrepancies is that the transgenics in the various animals have varying degrees of expression. More specifically in the context of animal models, these investigations establish a situation whereby the early stages of pathogenesis are determined by the expression of mutant SOD1 in neurons whereas expression in non-neuronal cells is critical to control ALS development. To understand how ALS affects the three components of the tripartite synaptic synapse, it is crucial to study their distinct roles in the chain of events that culminates in muscle fiber denervation. Even though there is a lot of information on motor neuron degeneration in ALS pathogenesis, few research have examined the changes that take place at the NMJ's motor nerve terminals.

5. CONCLUSION

In neuromuscular junction (NMJ) innervation in SOD1 mice was lost at 40 days of age in this research based on the structural properties of the NMJ in WT mice and SOD1 mice. At 60

and 90 days, there was significant structural damage to the NMJ and loss of motor axons. We found structural alterations in presynaptic and postsynaptic structures, as well as a reduction in synaptic vesicles, as a result of our research. To test whether these systemic alterations may be a therapeutic target, we looked at the underlying chemical mechanisms that cause them.

ETHICAL APPROVAL

ALL ANIMAL EXPERIMENTS WERE CARRIED OUT IN ACCORDANCE WITH THE GUIDELINES FOR THE MANAGEMENT OF EXPERIMENTAL ANIMALS FORMULATED BY THE MINISTRY OF SCIENCE AND TECHNOLOGY OF THE PEOPLE'S REPUBLIC OF CHINA AND THE INTERNATIONALLY ACCEPTED GUIDELINES ISSUED BY THE NATIONAL INSTITUTE OF HEALTH.

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