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Ghulam Hussain

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Ecole Doctorale Des Sciences De La Vie Et De La Santé (ED 414)

**Inserm U1118 Mécanismes Centraux et Périphériques de la
Neurodégénérescence**

THÈSE

Présentée par :

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Soutenue le : **09 Juillet 2013**

Pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : **Neuroscience**

**Rôle de la stearoyl-coA desaturase-1 dans le
maintien de l'activité musculaire: étude d'un modèle
lésionnel pour la compréhension des altérations
métaboliques caractéristiques de la
sclérose latérale amyotrophique**

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ABBREVIATIONS

ABCA1	ATP-binding cassette transporter A1
ACC	Acetyl-CoA Carboxylase
ALA	α -Linolenic Acid
ALS	Amyotrophic Lateral Sclerosis
AMPK	AMP-activated protein kinase
ANG	Angiogenin
ANG	Angiogenin
ASK1	Apoptosis Signal-regulating Kinase-1
ASO	Antisense Oligonucleotide
BAT	Brown adipose tissue
BDNF	Brain-derived neurotrophic factor
BMI	Body Mass Index
C9ORF72	Chromosome 9 open reading frame 72
CaMKII α	Calcium Calmodulin dependent Kinase II alpha
cAMP	cyclic Adenosine Monophosphate
CE	Cholesteryl Esters
CHO	Chinese hamster ovary
ChREBP	Carbohydrate Response Element Binding Protein
CNTF	Ciliary Neurotrophic Factor
CO	Corn oil
CPT1	Carnitine Palmitoyl Transferase-1
DAG	Diacylglycerol
DGAT	Diacylglycerol Acyltransferase
DNL	De Novo lipogenesis
EFAs	Essential Fatty Acids
EGF	Epidermal Growth Factor
ER	Endoplasmic Reticulum
FALS	Familial Amyotrophic Lateral Sclerosis
FAS	Fatty Acid Synthase
FFA	Free fatty acid
FGF-1	Fibroblast Growth Factor-1

FMRP	Fragile X Mental Retardation Protein
FMRP	Fragile X mental retardation protein
FTD	Frontotemporal dementia
FUS	Fused in Sarcoma
GC	Gas chromatography
GDNF	Glial-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
GRN	Progranulin
HPLC	High performance liquid chromatography
Hsp70	Heat Shock Protein 70
IGF	Insulin-like Growth Factor-1
IMTAG	Intramyocellular Triacylglycerol
IR	Immunoreactive
LA	Linoleic Acid
LBHIs	Lewy body-like hyaline inclusions
LMNs	Lower Motor Neurons
LXR	Liver X receptor
MCK	Muscle Creatine Kinase
MF	Metformin
MGF	Mechano-growth factor
MND	Motor neuron disease
MRI	Magnetic resonance imaging
MUFAs	Monounsaturated Fatty Acids
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NFG	Nerve Growth Factor
OA	Oleic Acid
OPTN	Optineurin
OPTN	Optineurin
PDK	Pyruvate Dehydrogenase Kinase
PGC1- α	Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
PLs	Phospholipids

POAG	Primary Open-Angle Glaucoma
ROS	Reactive Oxygen Species
RRM2	RNA recognition motif 2
RXR	Retinoid X receptor
SALS	Sporadic Amyotrophic Lateral Sclerosis
SDH	Succinate dehydrogenase
SFAs	Saturated Fatty Acids
SIRT1	Sirtuin
SMSC	Skeletal Muscle Satellite Cells
SOD	Superoxide dismutase
SPT	Serine Palmitoytransferase
SRE	Sterol Responsive Element
SREBP	Sterol Responsive Element Binding Protein
STAU1	Staufen
TAG	Triacylglycerol
TARDP-43	TAR (trans-activation response) DNA Binding Protein-43
TDZs	Thiazolinediones
TLR2	Toll-Like Receptor-2
TLS	Translocated in Liposarcoma
TMDs	Transmembrane domains
TNF- α	Tumour Necrosis Factor-alpha
UBQLN	Ubiquilin
UBQLN2	Ubiquilin 2
UCP-1	Uncoupling protein-1
UCP-1	Uncoupling protein-1
UMN	Upper Motor Neuron
UMNs	Upper Motor Neurons
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
XPD	Xeroderma pigmentosum factor D

INTRODUCTION

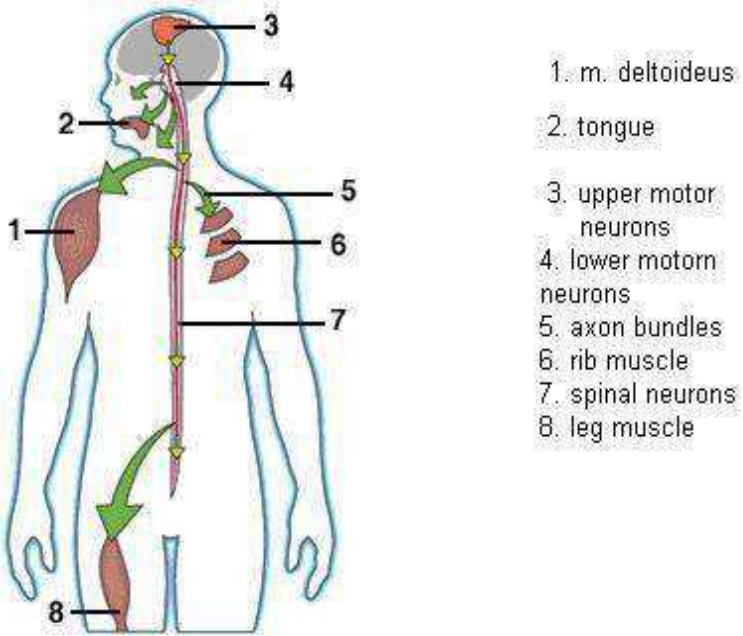
1. Amyotrophic Lateral Sclerosis (ALS):

ALS is a fatal motor neuron disease that relentlessly progresses to damage the upper motor neurons (UMNs) in the motor cortex and lower motor neurons (LMNs) in the spinal cord and brainstem (Fig.1a). This selective loss of upper and lower motor neurons results in muscle atrophy and weakness. This muscle atrophy causes generalised corporal and muscular weakness lastly respiratory failure that leads to demise (Fig.1b). About 90% of all reported ALS cases are sporadic with unknown cause while the rest of the 10 % are inherited or familial. Approximately 2% of familial ALS and 0.3% of sporadic ALS patients possess mutations in free radical scavenger Copper/Zinc superoxide dismutase 1 enzyme called SOD1.

The loss of motor neurons and corticospinal tract degeneration are the most prominent neuropathological features of ALS. LMNs in the brainstem and spinal cord are lost most frequently but UMNs loss in primary motor cortex is also inevitable in this disorder. Both Sporadic and familial forms of ALS share common pathological characteristics and loss of lower motor neurons. In some cases corticospinal tracts are also affected in FALS. In some reports, degeneration has been observed in the middle root zone of the posterior column, Clarke's nuclei and spinocerebellar tracts.

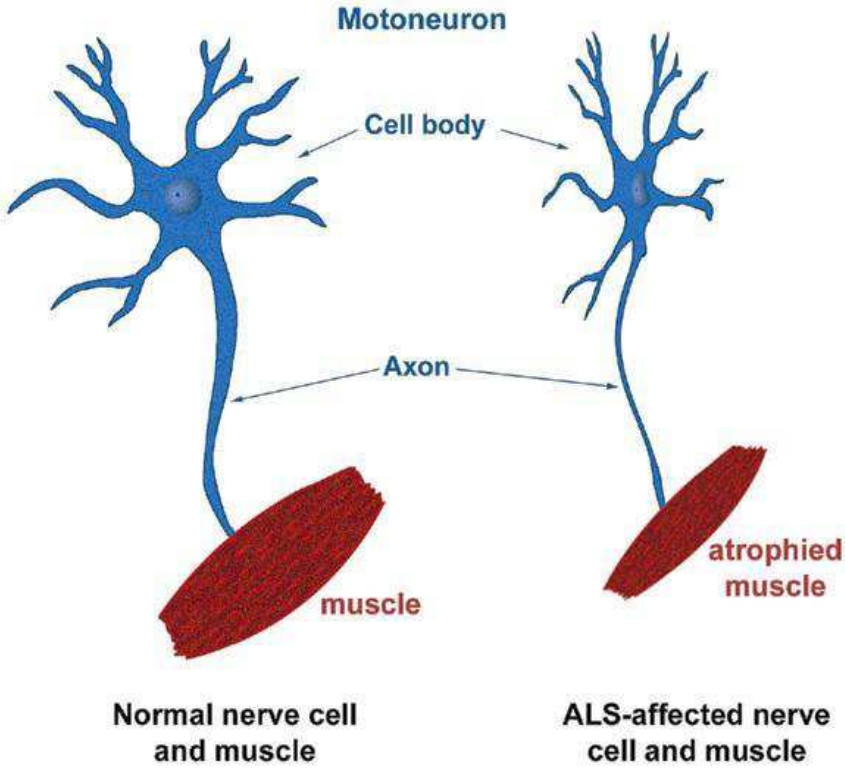
Cytoplasmic inclusions represent a hallmark of both sporadic and familial ALS while Bunina bodies and ubiquitin-immunoreactive (IR) skein-like or globular inclusions comprising protein aggregates are found in SALS. In case of FALS ubiquitin-immunoreactive hyaline and conglomerate inclusions are observed (Maekawa et al., 2009).

Fig. 1a:



http://www.steadyhealth.com/articles/Diagnosed_with_ALS_Frequently_Asked_Questions_a98.html

Fig. 1b:



<http://schoolworkhelper.net/amyotrophic-lateral-sclerosis-lou-gherigs-disease/>

2. Epidemiology:

The epidemiological data about ALS is much complicated to collect than that of other diseases. Different factors such as the true onset date, long duration between onset and clinically manifested disease symptoms and less reported or unreported cases due to unawareness among the people are the major causes. There is a long delay between the onset of disease and appearance of clinically distinguishable and diagnosable characters. This prodromal period of the disease may indicate the redundancy of neurons. Depending upon the data from various studies, different causative mechanisms of ALS have been suggested (Fang et al., 2008; Sutedja et al., 2009). The data about the incidence of ALS is very limited and the available data have highlighted that the incidence in European ethnic population is about even, 2.16 per 100 000 person-years. ALS is found mostly all over the world but the data about its incidence in most parts of the world is still lacking. The prevalence of ALS is not uniform in all geographical regions of the world. Some areas in the world such as Guam inhabited by the Chamorro people have historically had a high incidence of ALS. The people of Guam suffer from a disease called Lytico-Bodig disease which is a combination of symptoms similar to ALS, dementia and Parkinsonism. The prevalence of this disorder shows gender based inclination; the prevalence of ALS in men is higher (3.0 per 100 000 person-years; 95% CI 2.8–3.3) than in women (2.4 per 100 000 person-years; 95% CI 2.2–2.6). In FALS gender difference is of little importance. The overall population-based lifetime risk of ALS is 1:400 for women and 1:350 for men. The onset age of disease is different for familial and sporadic ALS. For sporadic ALS the peak age of onset is 58–63 years and 47–52 years is for familial ALS, the possibility of incidence becomes minimum after 80 years of age (McCombe and Henderson, 2010; Kiernan et al., 2011).

3. Molecular Genetics:

Although the phenotypic characters of ALS patients are apparently similar but there are some studies on European population that demonstrate the subtle differences among the registered patients' data. Population based studies have suggested that the people from mixed ancestors are less prone to ALS (Kiernan et al., 2011).

According to the available evidence, 5-10% of reported cases of ALS are familial ALS with inherited transmission of diseases by a Mendelian pattern. For familial ALS, there are 16 genes or loci identified to date. The name and functions are listed in table and detail functional information is described below (Table. 1).

Table. 1: Common genes involved in ALS

Gene	Locus	Protein	Found in cellular inclusions	ALS subtype	Other
Autosomal dominant FALS genes also implicated in SALS					
<i>SOD1</i>	21q22.1	Cu/Zn superoxide dismutase (SOD)	+	ALS1	Can be recessive in FALS
<i>FUS</i>	16p11.2	Fused in sarcoma (FUS)	+	ALS6	Can be recessive in FALS
<i>ANG</i>	14q11.1	Angiogenin (ANG)		ALS9	Autosomal Dominant or Haploinsufficient
<i>TARDBP</i>	1p36.22	TAR DNA Binding Protein-43 (TDP-43)	+	ALS10	
<i>OPTN</i>	10p13	Optineurin	+	ALS12	Can be recessive in FALS
<i>C9ORF72</i>	9p21	C9ORF72	?	'ALS-FTD'	Newly characterized
Autosomal dominant FALS genes					
<i>ALS3</i>	18q21	ALS3		ALS3	
<i>SETX</i>	9q34.13	Senataxin		ALS4	Can cause juvenile onset
<i>ALS7</i>	20p13	ALS7		ALS7	
<i>VAPB</i>	20q13.33	VAMP-associated protein B	+	ALS8	Can cause juvenile onset
<i>FIG4</i>	6q21	Phosphoinositide 5-phosphatase		ALS11	
<i>VCP</i>	9p13.3	Valosin-containing protein		ALS14	
Autosomal recessive FALS genes					
<i>ALS2</i>	2q33.1	Alsin		ALS2	Can cause juvenile onset
<i>SPG11</i>	15q15.1	Spatacin		ALS5	Can cause juvenile onset
X-linked dominant FALS gene					
<i>UBQLN2</i>	Xp11.2	Ubiquilin-2	+	ALS15	Can cause juvenile onset
Other genes					
<i>ATXN2</i>	12q24.1	Ataxin-2		ALS13	Increases ALS susceptibility

(Pratt et al., 2012)

3.1 SOD1 (Superoxide Dismutase-1):

Super oxide dismutase-1 (SOD1) is a member of an enzyme family which catalyzes the dismutation of superoxides into oxygen and hydrogen peroxide and figures out a primary defense mechanism against reactive oxygen species (ROS). There are 3 well documented members of the dismutase family in human; SOD1 (Cu/Zn-SOD), SOD2 (Mn-SOD) and SOD3 (EC-SOD), localized in cytoplasm, mitochondria and extracellular space (Zelko et al., 2002; Miao and St Clair, 2009). Nearly all aerobic organisms can synthesize dismutase enzymes to combat the toxic effect of superoxide and nitric oxides produced as a result of metabolism. Any dysregulation in the balance or neutralization of these by-products of oxidative metabolism can lead to the enhanced and toxic levels of reactive species of metals. The biological level of ROS is under the strict control of enzymatic activity and synthesis of the SOD and nitric oxide and their synthase enzymes. Moreover, these superoxide and nitric oxide can lead to the initiation of peroxidation of lipids and arachidonate (Shin et al., 2009). In eukaryotes, SOD is considered as a master regulator of oxygen radicals with relevance to a number of pathologies and functions such as neurodegenerative disorders, aging and cancer.

Superoxide Dismutase 1 (SOD1) was discovered by Joe M. McCORDS and Irwin FRIDOVICH in 1969. The gene SOD1 which is conserved from bacteria to human, encodes a cytosolic enzyme Cu/Zn SOD that catalyzes the dismutation of toxic superoxide radical ($O_2^{\cdot-}$) and converts it into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (McCord JM, 1969; Pratt et al., 2012). About 150 mutations in SOD1 have been reported which make it a significant causative factor for FALS. The mutant SOD1 accounts for 20 % of FALS cases (that ranges from 2.5% to 23.5%) and in the case of sporadic ALS, it ranges from 0.44% to 7% (Van Es et al., 2010; Pratt et al., 2012). The expression of SOD1 mutation is most often dominant and one copy of mutated allele is strong enough to induce disease. The homozygosity or increased number of mutated alleles aggravates disease and shortens the age of onset (Hayward et al., 1998; Marucci et al., 2007). The only exception reported till date is D90A SOD1 (Asp90Ala- Alanine for Aspartate at position 90) mutation in this gene. This is the world most reported mutation in SOD1 linked to FALS as well as SALS in homozygous manner

(Andersen et al., 1995). Both dominant and recessive functions of this mutation have been reported by various studies; homozygous or heterozygous expression of this mutation is sufficient to trigger the disease onset (Andersen et al., 1996, 1997; Al-Chalabi et al., 1998; Jonsson et al., 2002; Broom et al., 2009; Luigetti et al., 2009; Giannini et al., 2010). It is thought that the mutations in the SOD1 gene exhibit their effect by gain of function. Some studies show that a mutation without affecting the enzymatic activity or enhancing the gene expression can lead to the pathogenesis (Gurney et al., 1994) but absence or deletion of SOD1 seems not to be lethal. The mice with deleted SOD1 gene developed normally and exhibited no signs of denervation at 6 months age. These mice showed enhanced loss of neurons following a nerve injury. The active functional importance of this enzyme is necessary to combat the stress but it is not inevitable for survival (Reaume et al., 1996). The mutations noted in the SOD1 gene have been found not to be enzyme active site restricted (Deng et al., 1993).

The mutations in SOD1 are responsible for the formation of toxic inclusions in neurons and astrocytes which can be observed even at pre-symptomatic stage of disease in mouse model of ALS. It is postulated that these misfolded species of proteins play a key role to induce neurodegeneration (Bruijn et al., 1997; Prudencio and Borchelt, 2011; Pratt et al., 2012). The aggregates of misfolded protein of SOD1 are thought to be a result of interaction between SOD1 mutant protein and other proteins. A well-defined protein is Hsp70 and a number of studies have demonstrated that aggregates of SOD1 are associated with Hsp70. It is a notion that at initial stage neurons are able to avoid this aggregation by cytosolic soluble mechanism but at later stage aggregation overcomes this ability and results in neuron demise (Watanabe et al., 2001; Wang et al., 2009; Zetterström et al., 2011). Some pieces of evidence have shown the co-localization of 14-3-3 proteins with the aggregates of SOD1 in neuronal cells. Immunohistochemical analysis reveals the fact that mutant SOD1 protein shows high affinity for 14-3-3 proteins. The mice with mSOD1 gene show high immunoreactivity while mice wild type hSOD1 or non-transgenic mice show faint immunoreactivity against 14-3-3 proteins in aggregates (Okamoto et al., 2011). Some recent studies have found the strong immunoreactivity of SOD1 in non-ubiquitin small granular inclusions. These inclusions were not restricted to SOD1-

ALS but were also found in both SOD1 link FALS and non-SOD1 FALS, and similarly in non SOD1 sporadic ALS patients (Forsberg et al., 2010). It is important to note that aggregate formation by the interaction of SOD1 protein and others is observed only in the mutant case while normal SOD1 protein does not interact to result aggregate formation (Urushitani et al., 2006; Zhang et al., 2007).

The SOD1 mutations induced inclusions formation in astrocytes, microglia and oligodendrocytes have also been reported in both FALS and SALS (Fig. 2). These aggregates are sometimes observed in glial cells' nuclei. Aggregation is totally independent of SOD1 mutations presence or absence in ALS patients as ALS patients without SOD1 mutations also demonstrate the presence of aggregates. Therefore, it cannot be concluded that the aggregates observed in the ALS patients arise either from SOD1 mutations or from the complex and diverse cellular inclusions (Forsberg et al., 2011). Structural and enzymatic function analyses of SOD1 that includes the comparison of human SODs with other organisms for example bacteria have provided well illustrated information to evaluate the mutations found in this superoxide dismutase gene (SOD). Many groups have tried to elaborate the complex functional participation of SOD1 mutations in ALS pathogenesis and a number of mechanisms have been proposed (Danielsson et al., 2011; Grad et al., 2011; Pratt et al., 2012).

One of these proposed hypotheses is framework-destabilization. According to this hypothesis, every mutation in SOD can lead to the aggregation by unfolding proteins at local level. This destabilization and aggregation give deleterious results by interacting with other proteins (DiDonato et al., 2003). It has also been reported that such mutations that result framework-destabilization are also correlated with other neurodegenerative disorders and other diseases for example diseases that arise from the mutations in XPD helicase (Fan et al., 2008). The mutations in SOD1 are still elusive from their aggregative, destabilizing and disease causing mechanism aspects. In spite of all efforts, we cannot establish a relation between proteins aggregation propensity, destabilization and disease progression. The results from various studies do not corroborate their findings. One study suggests that aggregate propensity and instability are directly correlated with disease progression and survival. Other team reports that increased soluble aggregate formation and decrease stability of

immature apo SOD1 protein do not account for disease onset but other factors may play a role in this regard. We still lack confirmatory and solid data to suggest any concrete role of these structural and functional changes of SOD1 protein in the ALS progression at clinical level (Wang et al., 2008b; Prudencio et al., 2009; Byström et al., 2010; Vassall et al., 2011). It has been reported that irrespective of native species of protein, the immature apo SOD1 protein stability governs the survival in such way that this relation is directly proportional i.e., the more loss in stability, the more lower survival time (Lindberg et al., 2002; Rumfeldt et al., 2009).

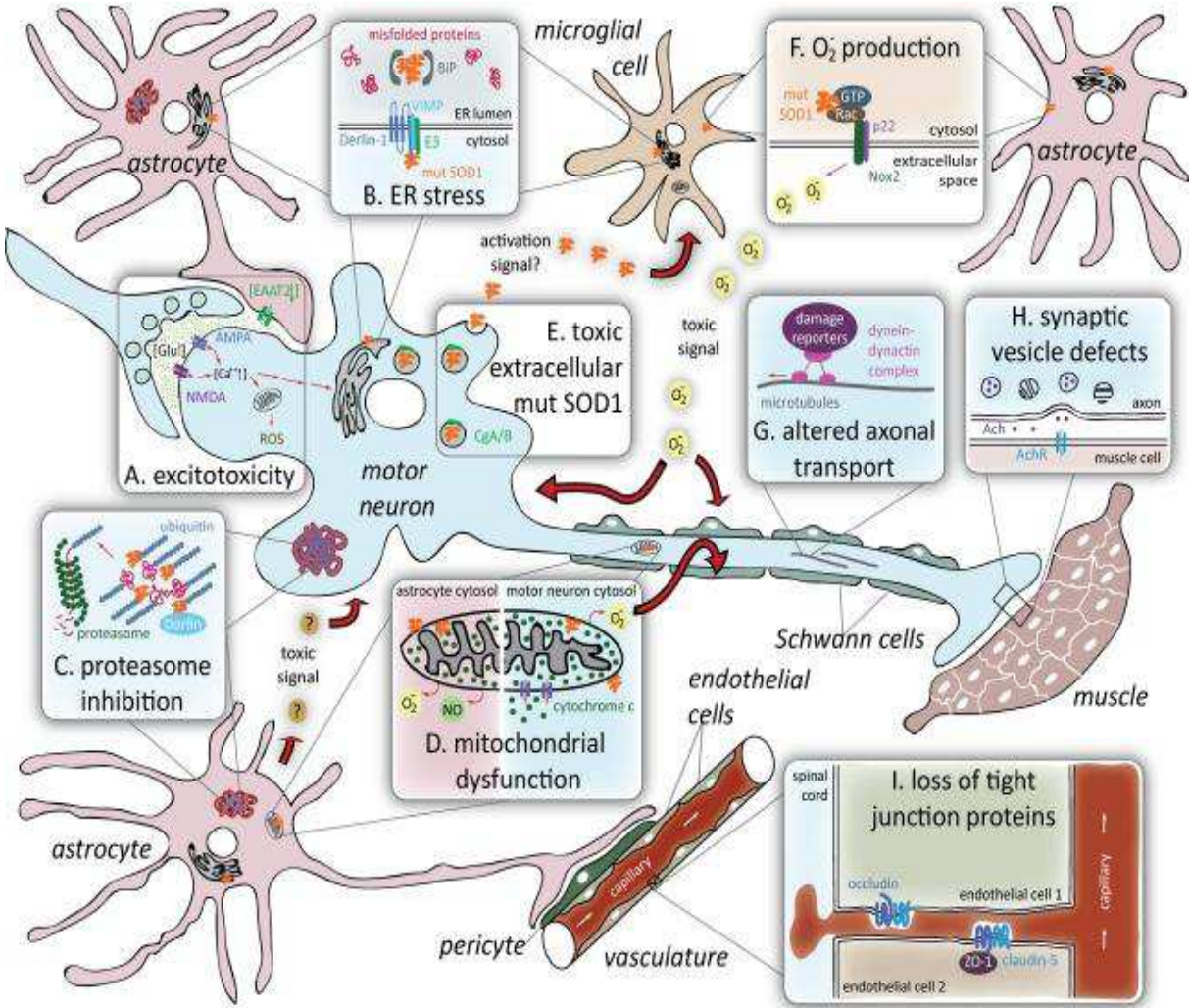
Conformational changes that may emerge from metal depletion or aberrant oxidization at post translational level in wild type SOD1 and as a result of mutation in mutant SOD1 recapitulate the same gain of toxic function *in vivo* and *in vitro* studies. Aberrant conformational abnormalities even in the wild type SOD1 may lead to pathogenesis. They increase kinesin-based fast axonal transport through p38 kinase activation and disclose a common mechanism of neurodegeneration in FALS and SALS (Bosco et al., 2010b). It is speculated that inability in establishing a correlation is a result of a large number of factors. These factors include the important role of metals in structure stabilization, aberrant oxidization, abnormal interaction of mutant SOD to other proteins and cellular organelles (Roberts et al., 2007; Sahawneh et al., 2010; Proctor et al., 2011; Furukawa, 2012; Pratt et al., 2012).

There have been many efforts to elucidate the molecular mechanism of mSOD1 involved in neuron degeneration. Following are the illuminated pathways by which the mutations in SOD1 protein lead to the fatal ALS. These may comprise protein-protein interaction, change in gene expression and modified cellular functions. The SOD1 mutant protein interacts with other cellular components to induce motor neuron death. These cellular components may include the induction of proteins responsible for any kind of stress for example Derlin-1. The dysfunction in SOD1 protein and Derlin-1 mediates neuron death by activating apoptosis signal-regulating Kinase 1 (ASK1) pathway (Nishitoh et al., 2008). SOD1-Rac1 interaction triggers NADPH oxidase-dependant superoxide ($O_2^{\bullet-}$) production which highlights another important function of SOD1 that SOD1 not only functions as catabolic cytosolic enzyme but also controls the production of superoxide ($O_2^{\bullet-}$) by interacting with Rac1 and inhibiting its GTPase activity

(Harras et al., 2008). Mutant SOD1 protein interaction with Hsc70 and Cu/Zn copper chaperone has been reported for aggregate formation for example Lewy body-like hyaline inclusions (LBHIs) a hallmark of progressively developing motor neuron disease (Kato et al., 2001; Wang et al., 2009).

The interaction between mutant SOD1 protein and transporter proteins in cells play a role in the retrograde transport of neurons. The retrograde transport plays an important role in the regeneration, response to stress or injury and neurotransmission. Chromogranin mediated secretion of mutant SOD1 and dysfunction of dynein dependant retrograde transport in axon are examples of such interactional activities. The mutation in dynein has been demonstrated to rescue the axonal transportation defects and extends life span of ALS mouse model (Kieran et al., 2005; Eschbach and Dupuis, 2011).

Fig. 2: Mechanisms of SOD1 toxicity



(Ilieva et al., 2009)

3.2 TARDBP-43:

TARDBP gene located on chromosome 1p36.22 encodes a 414 amino acids protein of 43 kDa called TDP-43 (Ou et al., 1995). The TAR DNA binding protein 43 that is commonly known as TDP-43 protein is present in cytosol and as well as in nucleus. Its functions mainly as a modulator of RNA/DNA binding, transcriptional regulator and most importantly participates in RNA processing (Da Cruz and Cleveland, 2011; Lagier-Tourenne et al., 2012; Pratt et al., 2012).

TDP-43 protein binds with both DNA and RNA. It is a DNA-binding protein because of having a sequence that facilitates nuclear export of DNA by interacting with (TG)_nT_m element in DNA. Similarly, it is also a RNA-binding protein that interacts with single-stranded RNA by binding with (UG)₆₋₁₂ motifs of RNA. In normal condition, TDP-43 is purely a nuclear protein while in pathological conditions it is delocalized in cytosol. TDP-43 is a unique protein that has been reported to bind with the mRNA of human low molecular weight neurofilament (hNFL) and stabilization of hNFL- mRNA is among its vast range of functions. This stabilization is done through interaction with the 3'UTR. In case of ALS, the delocalized TDP-43 results in NF aggregate formation (Strong et al., 2007).

In rodent model, depletion of TDP-43 gene reduced the synthesis of mRNA from pre-mRNA and altered the splicing of a wide range of mRNA transcripts. A few non-coding RNAs and gene transcripts with long introns were also among its targets. TDP-43 maintains a constant level of genes expressed in neurons by preventing the degradation of mRNA mediated by any dysfunction in this machinery. Its own expression is regulated by an autoregulation mechanism by which it binds directly to the 3'UTR of its own transcript and enhances splicing of intron. In this way a nonsense-mediated RNA degradation is triggered to control the expression of TDP-43 gene (Polymenidou et al., 2011; Colombrita et al., 2012).

TDP-43 protein functions as a neuronal activity responsive factor that regulates the neuronal plasticity. It is reported to manufacture various forms of nuclear substructures that facilitate the linkage of nuclear bodies of different types. In hippocampal neurons, TDP-43 protein functions as a neuronal activity-responsive factor in dendrites. It co-localizes with post-synaptic protein PSD-95 in the form

of RNA granules in somatodendrites and these granules also contain others RNAs for example; mRNA of β -actin and Calcium Calmodulin dependent Kinase (CaMKII α) in particular. The localization of TDP-43 granules with other RNA binding and mRNA transportor proteins for example Fragile X Mental Retardation Protein (FMRP) and Staufen 1 in neurons is enhanced by KCl stimuli (Wang et al., 2008a). Elevation of intracellular Zn induces fragmentation of TDP-43 leading to the cytoplasmic inclusion formation (Caragounis et al., 2010). There may be stressed induced nuclear TDP-43 depletion or fragmentation of long mRNA because some studies have shown the localization of TDP-43 with cytosolic stress granules (SGs) (Bentmann et al., 2012; Dewey et al., 2012; Parker et al., 2012). Aggregates promoting small fragments of TDP-43 protein contain c-terminal that uses partial RNA recognition motif domain 2 (RRM2) in ALS and FTLD. The functional and conformational stability in RRM2 is highly sensitive to acidic or saline conditions and modifications in acidic residue in this motive can alter the TDP-43 protein assembly (Shodai et al., 2012, 2013; Wang et al., 2013).

TDP-43 mutations are found in both familial and sporadic ALS and may contribute 6.5% in FALS and 0%-5% in SALS. The mutations found in this gene are inherited in both dominant gain of function and loss of function pattern. Till date, 40 mutations have been identified in TDP-43. In *in vitro* studies, mutant form of TDP-43 fragmented more readily than wild type. Neural apoptosis and developmental delay in the chicken embryo have also been reported due to a mutant form of TDP-43 (Kabashi et al., 2008; Rutherford et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008; Pratt et al., 2012). Moreover, the mutations in this gene enhance propensity to aggregate formation and toxicity (Johnson et al., 2009) and loss of function can also result toxic effects (Kabashi et al., 2010). In rodent model, the expression of mutant TDP-43 gene results ALS like pathology and mimics most of the symptoms for example spastic paralysis, inclusions in neurons and aggregates (Wegorzewska et al., 2009; Wils et al., 2010; Igaz et al., 2011). Similarly, dysregulation of TDP-43 gene in *Drosophila* affects synaptic transmission and motor neuron functions adversely (Diaper et al., 2013). The mutations found in TDP-43 cause neurodegeneration independent of SOD1 or FUS mutations (Mackenzie et al., 2007; Kühnlein et al., 2008; Vance et al., 2009). Sometimes, the mislocalization of TDP-43 is also associated with SOD1 misfolding (Pokrishevsky et al., 2012).

A form of dementia called frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and ALS have Ubiquitin-positive, tau- and α -synuclein-negative inclusions that are hallmarks of FTLD-U and ALS. It has been demonstrated that TDP-43 protein diminishes in nucleus in both diseases. The pathological form of TDP-43 was ubiquitinated and hyper-phosphorylated. Only the affected regions of the CNS; hippocampus, spinal cord and narcotics were found positive for TDP-43 inclusions (Arai et al., 2006; Neumann et al., 2006). These inclusions have been observed in neurons and other fellow cells such as glial cells in Guam ALS and SALS. In these inclusions, TDP-43 co-localizes with p62 and ubiquitin (Maekawa et al., 2009).

It has been found that progranulin gene (GRN) loss of function is a key factor for aggregate formation in TDP-43 mutations linked pathology of FTD (Baker et al., 2006; Cruts et al., 2006) . GRN gene encodes a secreted growth factor with a diversity of suggested functions in nervous system. One of the proposed functions performed by GRN includes long term survival of neurons. Although deficiency in GRN is directly linked to the malfunctioning of TDP-43 and aggregate formation in neurons but it is yet to be elucidated the relationship between the TDP-43 dysfunction and GRN decrease in expression (Ahmed et al., 2010). TDP-43 protein interacts with other proteins to participate in complex cellular mechanism essentially needed for cell survival. TDP-43 forms a complex with fragile X mental retardation protein (FMRP) and Staufen (STAU1) that finally affect the expression of SIRT1. TDP-43/FMRP/STAU1 complex regulates the mRNA level of SIRT1 which governs the DNA repair and cell survival. Dysfunction in any component of this complex may provoke apoptotic cell death (Yu et al., 2012). In the same way, RNA processing suggests the possible mechanism of neurodegeneration in ALS (Kim et al., 2010; Keller et al., 2012; Cirillo et al., 2013).

3.3 FUS/TLS:

Protein synthesis a vital process for cell survival and function depends on RNA processing. There are coding RNA and non-coding RNA in cell and their expression, processing (splicing) and transport are inevitable for normal function and differentiation of cells. In the last few years a special focus has been given to the RNAs role in neurodegenerative disorders. Since, TDP-43 and FUS/TLS are DNA/RNA binding proteins and play a key role in RNA processing for its normal

function, so these genes have been particularly focused. Failure in RNA processing yields abnormal protein synthesis that can alter cellular functions and finally maladies (Yang et al., 2010; Polymenidou et al., 2012; Renoux and Todd, 2012).

FUS/TLS was identified in 1993 as a part of fusion gene with the transcription factor CHOP. It is a 525-residue protein that consists of 6 potential N-linked glycosylation sites and 3 glycine clusters (Rabbitts et al., 1993; Dormann et al., 2010). FUS/TLS shares an extensive homology with EWSR1 sequence and translocates in Ewing sarcoma. FUS/CHOP fusion causes replacement of RNA-binding domain by a DNA-binding domain (Croizat et al., 1993). The genomic structure of FUS discloses that the gene consists of 15 exons making 11kb of genomic DNA with multiple glycine repeats at various locations (Aman et al., 1996). FUS protein is found in the nucleus and performs various functions in DNA and RNA metabolism. These functions include DNA repair, transcription regulation, RNA splicing and its export to the cytoplasm (Lagier-Tourenne and Cleveland, 2009; Vance et al., 2009; Lanson and Pandey, 2012). TLS protein couples with CREB-binding protein (CBP) and p300 histone acetyltransferase and inhibits their activities on target gene. In this way FUS functions as a key transcriptional regulatory sensor of DNA damage signals from non-coding RNAs (Wang et al., 2008c).

A vast set of information demonstrates that dysfunction in RNA metabolism due to mutation in FUS/TLS or TDP43 is a pivotal step in ALS and FTD. Any abnormality in FUS functions may lead to pathological condition particularly neurodegenerative disorders like ALS. Expression of mutant FUS gene in mice mimics pathological symptoms while in zebra fish loss of FUS also results neurodegeneration (Kabashi et al., 2011; Verbeeck et al., 2012). TDP-43 and FUS/TLS inclusions are mostly found in FTD and others proteinopathies (Snowden et al., 2011) but this is not the case with all ALS cases. Co-localization of mutant forms of proteins in cytosol is a distinguished feature of FTD (Neumann et al., 2009, 2011). It was suggested that FUS or TDP-43 positive inclusions were absent in ALS patients. But in recent studies these inclusions have also been observed in ALS patients (Kwiatkowski et al., 2009; Lashley et al., 2011). It is interesting to know that some but not all FTD-U patients are diagnosed with ALS at lateral stages. This is yet unknown how some patients of

FTD with the same mutation in FUS, develop ALS after some time and other do not (Mackenzie et al., 2010).

Instead of FALS, there are some studies that show the mutations in FUS gene are also present in SALS (Chiò et al., 2011). The FUS mutations caused ALS is severe, early onset and with short life span (Sproviero et al., 2012), this early onset and disease severity are sometimes mutation type dependent (Mackenzie et al., 2011) . Mutations in FUS account for a small proportion of SALS that is about 1% (Lai et al., 2011). Juvenile ALS is a rare form of ALS and the age of onset is early of 25 year and *de novo* mutations in the FUS gene have been found to yield juvenile SALS in various ethnic groups (DeJesus-Hernandez et al., 2010; Zou et al., 2012).

Till date, 50 mutations in the FUS gene have been reported that cause amyotrophic lateral sclerosis (ALS). The mutations in this gene perturb DNA and RNA binding properties of this protein that lead to failure in DNA binding ability, RNA processing and transportation features of FUS. These functional abnormalities have been found more prominent in motor neurons and failure in RNA transportation causes aggregates formation a hallmark of some neuropathies ([Http://ghr.nlm.nih.gov/gene/FUS](http://ghr.nlm.nih.gov/gene/FUS), 2013). Most of the mutations in FUS/TLS have a dominant way of expression but some with recessive or loss of function have also been documented for example H517Q (Kwiatkowski et al., 2009; Kabashi et al., 2011; van Blitterswijk et al., 2013). The FUS mutations contribute 3%-5% cases of FALS and 1% cases of SALS and immunoreactive inclusions results from the mutant forms of FUS genes are a common feature of SALS and nonSOD1 FALS (Deng et al., 2010; Kabashi et al., 2011; Mackenzie et al., 2011; Pratt et al., 2012).

About 90% nonSOD1 ALS patients are positive for TDP-43 inclusions (Neumann et al., 2006) while remaining 10% nonSOD1 ALS patients have FUS positive inclusions (Neumann et al., 2009). Mislocalization of FUS/TLS or TDP-43 to cytosol can result the misfolding of endogenous human wild-type SOD1. Sometimes, wild-type SOD1 misfolding is observed in motor axons of the spinal cord and in the cytosol of FUS or TDP-43 ALS patients. This misfolding of SOD1 increases with increased expression of wild-type TDP-43 but over expression of wild-type FUS was found effectless against wt-SOD1 misfolding (Pokrishevsky et

al., 2012). It has been demonstrated by one study that RNA/DNA binding nuclear proteins of FUS/TLS or TDP-43 cause neurodegeneration by interacting with Survival Motor Neurons (SMN) complex that is considered to be responsible for the survival and maintains other important cellular issues (Yamazaki et al., 2012). Moreover, following a stress mutant FUS protein co-localizes with stress granules in mice and zebra fish that suggests a role of stress in diseases (Bosco et al., 2010a; Verbeeck et al., 2012; Takeuchi et al., 2013).

The effect of mutations and malfunctioning of FUS/TLS gene is not limited to the neurons, but it also affects other cells in both sporadic and familial ALS. The FUS positive cytoplasmic inclusions have also been observed in neuron supporter cells for example glial cells in the CNS (Hewitt et al., 2010).

3.4 C9orf72:

C9orf72 gene located on chromosomes 9 i.e., at locus 9p21, encodes a cytoplasmic protein found in neurons and synaptic regions of neurons. The functions of this protein are not well elucidated till date but its role in pathogenesis of neurodegenerative disorders such as ALS has been recently established. In 2011, it was reported independently by two groups that *C9orf72* was found closely linked to the dominant cases of ALS and ALS/FTD. They reported a substantial increase in hexanucleotide repeat (GGGGCC) in an intron of *C9orf72* gene. Minimum repeat size of 30 repeats is thought to be sufficient to cause disease.

The prevalence of *C9orf72* mutations causing ALS varies geographically and it was found highest in Greek patients (Majounie et al., 2012; Mok et al., 2012b; Sabatelli et al., 2012). The abnormality in the function of this gene is found in 24%-46% of FALS and it accounts for 4%-21% of SALS cases. In a study concerning European population, about 40% of FALS cases showed a mutation in this gene. Paternal and maternal transmission of this disease was 49.1% and 50.9% respectively. Onset age for diseases was shortened by 7 years in children. The parents had 62.8 years average age of onset while in children it was 55.8 years and it was mostly bulbar onset. The survival of *C9orf72* ALS patients also differed from other genes ALS after onset of disease. It was 3.2 years lower than TDP-43 ALS and 1.9 years longer than FUS ALS (Chiò et al., 2012; Cooper-Knock

et al., 2012). This high frequency of this gene makes it the most common factor provoking ALS pathogenesis.

C9orf72 caused pathology overlaps some other disorders adding more complexity to the understanding of the mechanism. ALS/FTD is a more common case but Parkinsonism and some other disorders have also been reported. The increase in repeat has been observed in FTD and ALS patients along with neuronal inclusions but the mechanism is yet to be elucidated (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Ferrari et al., 2012; Pratt et al., 2012).

In case of pathological mutation, this protein is localized in nucleus instead of cytoplasm and causes aberrant RNA splicing. Some researchers propose toxic gain of function by RNA due to failure in alternative splicing (Van Blitterswijk et al., 2012). Till date, HREM is considered the most common mutation in C9orf72 gene in European ethnic groups (Smith et al., 2012). MRI scans revealed the loss of gray and white matter in the frontal lobes and occipital lobe atrophy. It is suggested that a single nucleotide polymorphism on chromosome 9 found in all cases shows the same founder of this phenomenon (Boxer et al., 2011; Murray et al., 2011; Boeve et al., 2012; Brettschneider et al., 2012a; Hsiung et al., 2012; Mok et al., 2012a; Stewart et al., 2012).

In a case study of a family with FLD/ALS, no mutation was found in the exons of known genes for ALS or FTD diseases. This study bore a new idea that pathogenic changes in this family affect the non-coding DNA and diseases is caused by alterations in gene expression or protein synthesis (Pearson et al., 2011).

3.5 OPTN (Optineurin):

In human OPTN gene encodes a protein of 577 amino acids with coiled-coil domains and a C-terminal zinc finger. OPTN is a phosphoprotein that is expressed ubiquitously in all tissues but retinal ganglion cells (RGCs) are highly positive for its presence. At the time of identification, OPTN was considered as a NF- κ B essential modulator (NEMO) related protein due to 53% sequence homology with NEMO. Tumour necrosis factor alpha (TNF- α) enhances the expression of OPTN. OPTN is a negative regulator of TNF- α mediated NF- κ B activation. It binds to a ubiquitinated receptor-interacting protein that causes

over expression of the OPTN and creates a negative feedback loop to inhibit NF- κ B. NEMO and OPTN shares functional homology and are considered as competitors in functional point of view. It plays an important role in vesicular trafficking and organization of the Golgi apparatus by its interaction with myosin VI and Rab8. It also interacts with huntingtin, TANK-binding kinase 1 (TBK1), metabotropic glutamate receptors (mGluRs) and transcription factor IIIA. It controls mGluR signalling in striatal neurons and participates in autophagy by phosphorylating autophagy receptors (Anborgh et al., 2005; Ying et al., 2010; Sakaguchi et al., 2011; Wild et al., 2011; Pratt et al., 2012).

Primarily OPTN gene is a causative factor of an eye disease called POAG (Primary Open-Angle Glaucoma), a worldwide blindness disease. OPTN mutations account for 3.5% SALS and 1.2% FALS cases. 12 mutations with gain of function in dominant condition and with loss of function in recessive condition have been reported till date (Swarup and Nagabhushana, 2010; Del Bo et al., 2011). Both SALS and FALS, show the colocalization of OPTN with FUS and TDP-43 inclusions in neurons. The co-localization of OPTN with TDP-43 and FUS is not observed in other neurodegenerative disorders. In SOD1-ALS cases the presence of OPTN in the basophilic inclusions was also detected (Maruyama et al., 2010; Hortobágyi et al., 2011; Ito et al., 2011a).

In ALS, the inhibitory function of the OPTN is lost due to mutation and causes upregulation of NF- κ B a cell death inhibitor complex. NF- κ B has been reported upregulated in motor neurons of ALS patients. The overexpression of OPTN by upregulated NF- κ B in SALS may lead to motor neurons demise (Pizzi and Spano, 2006; Maruyama et al., 2010; Maruyama and Kawakami, 2012). It can be explained that upregulated OPTN inhibits the protective effect of E3-14.7K against cell death that results from TNF- α receptor 1 (TNFR1) overexpression (Zhu et al., 2007).

The true mechanism and participation of this gene in ALS pathogenesis is to be elucidated as the controversial results are continuously emerging with OPTN co-localization. In some studies, SOD1-ALS models were found positive for the OPTN presence in inclusions but other team reported totally opposite results at the same time (Deng et al., 2011a; Ito et al., 2011b).

3.6 ANG (angiogenin, ribonuclease, RNase A family, 5):

The angiogenin is a 14.1 kD protein encoded by ANG gene located at 14q11.2 cytogenetic location and is a member of pancreatic ribonuclease A superfamily and RNase. ANG plays an important role in cell proliferation, angiogenesis and neovascularisation. Although ANG was initially identified as an angiogenesis provoking agent in tumour tissue but its physiological function analysis revealed that ANG is a key player in the host defence system. Human possesses only one ANG gene while there are 4 isoforms in mice and 2 in cattle. The precursor protein consists of 145 amino acids that yields isoformic proteins of mature ANG4, ANG1 and ANG of 120, 121 and 132 amino acid residues respectively (Hooper et al., 2003). ANG stimulates angiogenesis and cell survival by interacting with other factors like acidic and basic fibroblast growth factors (aFGF, bFGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Inhibition of ANG by small interfering RNA (siRNA) masked the effects of other growth factors on cell proliferation. This suggests its vital role in the survival and proliferation of cell even in a condition when other angiogenic factors have high expression (Kishimoto et al., 2005).

The expression ANG was detected in the foetus and adult human spinal cord motor neurons, in endothelial cells and other tissues (Wu et al., 2007). Inflammation upregulates the expression of ANG in the liver and protein synthesis is detectably enhanced in serum. Ischemia significantly induces the expression of ANG in the brain and particularly in neurons in a rat model (Huang et al., 2009). Recently ANG has been reported as a candidate gene in ALS (Greenway et al., 2006). ANG in motor neurons is protective against excitotoxicity in PIK3/Akt kinase dependent manner. In case of motor neuron disorders, mutant ANG protein losses such protective effects. In ALS model mice SOD1 the life span and survival of motor neurons were ameliorated by extragenously supplied ANG and pathogenesis was reverted by this treatment (Kieran et al., 2008; Subramanian et al., 2008; Li and Hu, 2012).

The mutations in ANG gene cause loss of its normal functions and mutant proteins lack the angiogenic ability as a result of failure in its ribonucleolytic activity or nuclear translocation or both simultaneously. These mutations also change the thermal stability of ANG protein (Crabtree et al., 2007). 18 mutations have been reported in the ANG that cause loss of it's function. Most of the

reported cases are characterized with bulbar onset (Greenway et al., 2006; Pratt et al., 2012). There are evidences that show the association between Parkinson's disease (PD), ALS and AGN gene variants. So ANG functions as a link among various neurodegenerative disorders (Van Es et al., 2011). Motor neurons secreted ANG is endocytosed by glial cells in a clathrin mediated way by using heparin sulphate proteoglycans to mediate RNA cleavage. The syndecan 4 functions as ANG receptor for the selective uptake by astroglia. The mutant forms of ANG are also endocytosed by astroglia but they cannot mediate RNA cleavage. This piece of information extended our knowledge that ANG function is not limited to motor neurons, but it also involves other cells in the vicinity (Aparicio-Erriu and Prehn, 2012; Padhi et al., 2012, 2013; Skorupa et al., 2012).

3.7 UBQLN-2 (Ubiquilin-2):

The UBQLN-2 an X-chromosome linked gene at Xp11.23-p11.1 cytogenetic location encodes a protein called ubiquilin-2. It is a member of ubiquilin-like protein family that is characterized by a N-terminal ubiquitin like domain and a C-terminal ubiquitin associated domain presence. The two domains of this gene make it versatile in function such as the ubiquitin like domain binds to subunits in the proteasome and the ubiquitin associated domain binds to the polyubiquitin chains on the proteins to be degraded. The human UBQLN gene possesses no intron and has 4 isoforms with a unique protein. A recent study has reported the participation of UBQLN-2 in FALS in a dominant way of function. The mutations that have been reported to cause ALS were found on Proline rich (PXX) domains. The PXX domains of UBQLN-2 are for its specific interaction with other proteins for degradation and complex formation. Skein-like inclusions positive for ubiquilin-2 were observed particularly in the spinal cord and hippocampus of UBQLN-2 ALS patients. These inclusions were also positive for TDP-43, OPTN and FUS but not for SOD1. About 20% UBQLN-2 ALS patients develop dementia at a later stage. Since ubiquilin is involved in protein degradation, so the reduced level of UBQLN-2 in ALS patients is self-explanatory for inclusions formation (Deng et al., 2011b; Pratt et al., 2012). The overlap of UBQLN-2 pathology with FUS, TDP-43, C9ORF72 and OPTN pathology is also attracting attention (Brettschneider et al., 2012b; Fecto and Siddique, 2012; Synofzik et al., 2012; Williams et al., 2012).

4. Cellular origin of ALS: a multicellular pathology/disease:

For a long time, it has been considered that ALS is intrinsic to motor neurons due to limited molecular and cellular techniques and related information. In fact, it was strongly believed that autosomally inherited mutant SOD1 protein toxicity and related factors for example, oxidative stress and apoptosis, was cell autonomous and restricted to motor neurons solely. Some studies of the last few years have shattered this idea of the sole involvement of motor neurons and have opened new horizons in this scenario.

A set of evidences has demonstrated that ALS is not intrinsic to motor neurons only but its neighbouring cells also contribute their part (Fig.3). They play their role either in aggravating or ameliorating the damage to motor neurons caused by genetic, environmental or physiological factors. The targeted cells specific expression of mSOD1 has been intensively studied to elucidate the origin of pathology and essential cellular elements responsible for worsening or ameliorating the disease. A series of experiments has been performed in SOD1^{G37R} mice controlled by the promoter of the prion gene (PrP-SOD1^{G37R}). The transgene is highly expressed in neurons, astrocytes and skeletal muscle, these mice developed a disease very similar to ALS (Wang et al., 2005a; Ilieva et al., 2009). This indicates that the motor axis as a whole is necessary for the appearance of the full clinical picture of the disease.

Now it is believed that ALS is a multicellular disease involving an active participation of motor neurons and other neighbouring cells. It all became possible due to the recent advancement in the fields of molecular and cellular biology.

4.1 Motor neuron:

The hypothesis of ALS as a multicellular pathology emerged in early 2000s, when two groups lead by Pramatarova et al., 2001 and Lino et al., 2002, studied the specific over expression of mutated SOD1 in motor neurons exclusively to find out whether they could have pathogenesis. The systemic introduction of mutant SOD1 resulted in general ALS but the mice with over expression of mutant SOD1 restricted to neurons failed to develop any sign of neurodegeneration even after 1 and half year. These studies suggested that only motor neurons specific mSOD1 is not sufficient to yield pathology and thus other cells may also involve in ALS pathogenesis (Pramatarova et al., 2001; Lino et al.,

2002). But these studies cannot exclude the involvement of motor neurons in this pathological process. A more recent study, however, showed a degeneration of motor neurons could be observed in such animals, but the results are in very advanced age mice, and are questionable (Jaarsma et al., 2008). One possible reason may be the use of Thy1 promoter in this study and supposed explanation may be the very low expression level to reach a threshold level needed for arresting normal functions (Liu et al., 2004). Evidence that alteration of several cell types is a reality in the SLA was then reinforced by the work undertaken by Clement et al. They generated chimeric mice in which mSOD1 gene expressed in certain types of cells but not in others in the same tissue. They explored that the presence of wild-type cells in the immediate vicinity of a neuron carrying the mutant SOD1 prevents its degeneration and ameliorated the survival to some extent (Clement et al., 2003). In contrast, a wild type neuron juxtaposed a neuron with mutant SOD1 gene expression commences to show signs of degenerations for example aggregates formation. In a mouse with a ubiquitously expression of mSOD1 in motor neurons and microglia, the survival increased 20% by abolishing the expression of mSOD1 in glial cells by a genetic recombinant approach (Boillée et al., 2006; Yamanaka et al., 2008a). Sometimes the coincident threshold level of mSOD1 protein in motor neurons, astrocytes and muscles is reported to be sufficient to induce pathogenesis (Wang et al., 2005b).

The complex and vital interaction between neurons and astrocytes make this debate endless. In brief, these pieces of information establish the idea that only motor neuron with mutant SOD1 gene expression is not sufficient to induce ALS like symptoms but the controversy is still there due to ambiguous and insufficient data. Such kind of interesting but unclear results excited a large scientific community to study more systematically the impact and presence of mutated SOD1 in different tissues or cell population independently.

4.2 Astrocytes:

Astrocytes are the neighbouring cells of neurons in the CNS and constitute a large population greater than neuronal population. They are versatile in function as they provide trophic, structural and metabolic support to the neurons. They provide metabolic support by supplying energy metabolites and help in neurotransmitters recycling. Any impairment in astrocytes functions

impairs neuron function that may lead to death (Allaman et al., 2011). It has been found by *in vitro* studies that astrocytes secrete neurotrophic factors to affect neuron survival in various ways. These neurotrophic factors can provide protection against apoptosis by activating Akt pathway. Astrocytosis is the phenotypical change in astrocytes in case of any injury to the CNS. Similarly, enhanced expression of glial fibrillary acidic protein (GFAP), expression of cytoskeleton proteins, cell surface and matrix molecules, growth factors, cytokines, proteases and protease inhibitors are the indications of reactive astrocytes in response to the injury (Barbeito et al., 2004; Van Den Bosch and Robberecht, 2008; Vargas and Johnson, 2010).

Same is the reaction of astrocytes when there is neurodegeneration in ALS. During the last few decades, the focus of researchers has been on motor neurons as a central of initiation and to some extent the surrounding cells for example glial cells. Apoptosis is one of many processes observed in pathology and it emerged the notion of environmental factors that force neurons to target themselves being unable to sustain their health in hostile environment. Since astrocytes are responsible for homeostasis of neurons in the CNS and are closely related to neurons by making processes around neuronal cells, any functional abnormality in astrocytes can yield lethal effects on neurons (Sica, 2012).

Nrf2 is a transcription factor that regulates the antioxidant defence in astrocytes and influences the neighbouring neurons protection. The toxicity of astrocytes expressing mSOD1 to motor neurons was found reversed when Nrf2 was upregulated in astrocytes under glial fibrillary acidic protein (GFAP) promoter. Similarly the extended survival and delayed onset of diseases were the consequences when mSOD1 mice were crossed with GFAP-Nrf2 expressing mice (Vargas et al., 2008, 2013; Gupta et al., 2013).

Sometimes, astrocytes can adversely affect neurons. Neurons suffering from oxidative stress as a result of mutant SOD1, release fibroblast growth factor 1 (FGF-1) that activates astrocytes to secrete nerve growth factor (NGF) by inducing the accumulation of the FGF receptor 1 (FGFR-1) in astrocyte nuclei. The FGF1 is a protector of neurons in a case of spinal cord injury or axotomy but FGF1 mediated secretion of NGF by astrocytes stimulates apoptosis via the neurotrophin receptor p75NTR overexpression, thus withdrawing the protective

effect of FGF-1 (Cassina et al., 2005). This idea was further confirmed when wild type motor neurons were co-cultured with astrocytes expressing mSOD1 gene. The motor neurons were influenced by the toxic effect of mSOD1 expressed in astrocytes (Nagai et al., 2007; Marchetto et al., 2008; Staats and Van Den Bosch, 2009). These *in vitro* results very important in deciphering the mechanisms leading to motor neuron death are, however, offset by previous studies *in vivo*. Indeed, overexpression of mutant SOD1 under the control of glial fibrillary acidic protein (GFAP) promoter triggers a reactive astrogliosis without inducing motor neuron pathology in mice (Gong et al., 2000; Yamanaka et al., 2008b).

The controversy among these results can be interpreted by the essential and the new role of degenerating neural tissue in a medium that is a prerequisite to the neurodegeneration observed in ALS. Due to conflicting results, every step to explore fundamental mechanisms proved inconclusive.

4.3 Microglia:

Microglia, derived from the hematopoietic cell lineage and resident macrophages of CNS, represent the main and primary defence line of the CNS. They comprise 20% of the brain by the total population of glial cells. They make a surveillance system for CNS by making a dense arborisation of cell processes around neurons. They respond to the damages of various types such as invading microorganisms, serum microhemorrhage of blood vessels, abnormal protein synthesis, immunoglobulin-antigen complex and also remove cellular debris. The flexibility in changing their morphology from ramified to amoeboid, helps them to migrate limitlessly. They are highly sensitive to very small pathological changes in the CNS (Heneka et al., 2010; Kofler and Wiley, 2011; Lasiene and Yamanaka, 2011). There are many studies that demonstrate the impaired microglia in ALS due to their enhanced neurotoxicity as compared to the wild type microglia (Xiao et al., 2007). From the mechanistic point of view, it seems that the expression of mutant SOD1 in microglia facilitates microglial inflammatory response and induces neurotoxicity through TLR2 stimulation. This neurotoxicity is the result of neurotoxic cytokine and TNF- α upregulation mediated by NADPH oxidase dependent elevated synthesis of ROS (Liu et al., 2009; Li et al., 2011).

Several approaches have been used to explore the role of microglia as a site of initiation of pathology in ALS. Boillée and colleagues demonstrated by using a conditional mutant based on the Cre-Lox system that the mice with mSOD1^{G37R} crossed with Cre mice under the promoter of myeloid cells CD11b (leading to decrease the mutated SOD1 in microglia), their survival increased by almost three months (Boillée et al., 2006). However, despite the significantly increased lifespan in these mice, the onset of symptoms remained unchanged. These results suggest an important role of microglia in the final stages of the disease but not a cell type that induces symptoms. In addition, taking into account the promoter used (CD11b), it cannot be excluded that the beneficial effects of phenotypic improvement observed in these mice do not result from the inactivation of the transgene in other peripheral cell types. The involvement of other cell types such as astrocytes, oligodendrocytes and others cannot be totally excluded from this process.

These results were confirmed by another team with a different experimental approach. Using knockout mice for the transcription factor PU.1, necessary for the development of the myeloid cell line, the authors transplanted PU.1 ko mice with SOD1^{G93A} microglia from the bone marrow from mSOD1 mice. The PU.1 ko mice displayed no signs of neurodegeneration and muscle weakness. In contrast, when mSOD1/PU.1 ko mice were transplanted with microglia from a wild-type mice, they exhibited prolonged survival and retarded loss of motor neurons. The results confirm the previous study with improved survival, without modifying the age of onset of symptoms (Beers et al., 2006; Gómez-Nicola et al., 2013).

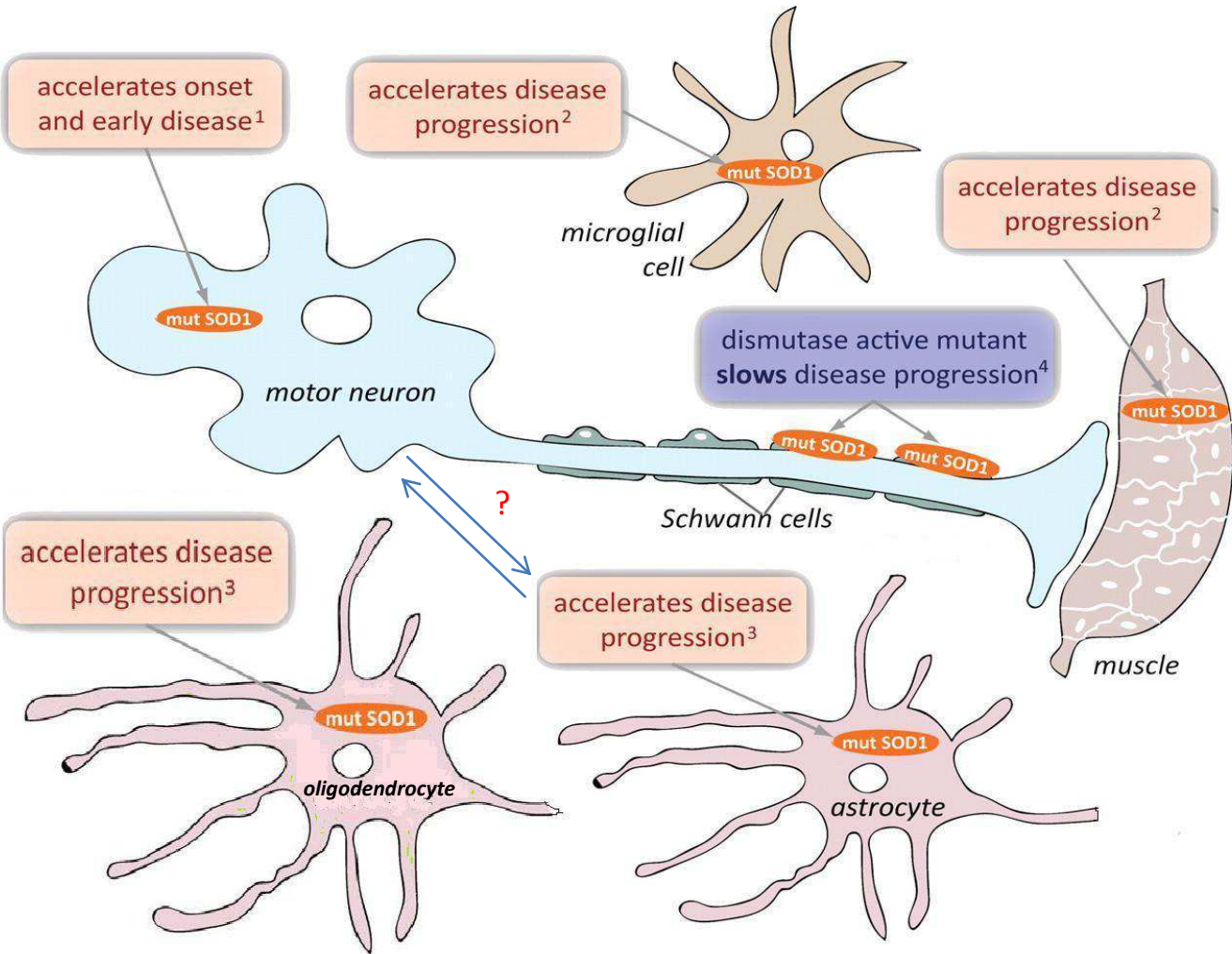
The controversy in the role of microglia is yet not limited to the above mentioned studies. In another study, the participation of proliferating microglia in motor neuron degeneration was assessed by generating CD11b-TK (mut-30); SOD1^{G93A} doubly transgenic mice. These double transgenic mice enabled the authors the elimination of proliferating microglia by administrating ganciclovir. The 50% reduction in reactive microglia in the lumbar spinal cord of these doubly transgenic mice proved effectless on motor neuron degeneration. This study negated the role of reactive microglia with mutant SOD1 expression as central contributors in neurodegeneration observed in ALS (Gowing et al., 2008). Whilst, another study reported contradictory results; elimination of proliferating microglia resulted in an exacerbated motor neurons demise (Audet et al., 2012;

Lerman et al., 2012). In a rat model of ALS, it has been shown that microgliosis appeared prior clinical symptoms appearance. The activity of microglia was found elevated in the spinal cord and peripheral sciatic nerve and authors found microglia and microphage encircling axons in ventral nerve root and sciatic nerve. This study demonstrated the involvement of microglia at the early stage of disease in ALS (Graber et al., 2010; Polazzi and Monti, 2010) and their activation is suggested to be correlated with disease progression (Brettschneider et al., 2012c). The extracellular mSOD1^{G93A} has no direct toxic effects to motor neurons rather it activates microglial cells for causing neurotoxicity by utilizing CD14 and TLR pathways (Zhao et al., 2010).

The interaction of motor neurons and glial cells is initially protective for neurons in the case of a stress. There are evidences that the motor neurons under stress do not undergo demise solely but they involve a well-orchestrated dialogue with microglia. However, various factors such as misfolded proteins of mSOD1, mitochondrial dysfunction and impaired axoplasmic flow increase stress and injury within motor neurons. As a result motor neurons release some signals that activate microglial cells. The microglia cells modify their physiological state from resident to reactive in turn and inverse their functions from anti-inflammatory and neuroprotective to pro-inflammatory and neurotoxic. This kind of signals from motor neurons result in the synthesis of reactive oxygen species and pro-inflammatory cytokines by microglial cells and add more stress to motor neuron (Appel et al., 2011; Rodríguez and Verkhratsky, 2011; Lewis et al., 2012; Liao et al., 2012).

Taken together, these results show how difficult it is to involve single cell type symptoms in the clinical forms of ALS. In fact, most of these studies indicate that the cell types targeted participate unquestionably the phenomenon of neurodegeneration, but none of them seems able by itself to trigger the onset of symptoms. Thus, the concept "non-cell autonomous" has emerged, it postulates that motor neuron degeneration is not autonomous, but it is the result of physiopathological phenomenon involving different stakeholders; particularly non-neuronal cells. It is in this context that we see now an assumption not limited to the CNS, but also includes peripheral tissues. Indeed, myocytes undergoing severe atrophy during disease also seem to participate in the global mechanism responsible for the motor neuron degeneration in ALS.

Fig. 3: Proposed effects of mSOD1 on ALS pathogenesis



(Modified from Ilieva et al., 2009)

5. Peripheral alterations: role and relevance of muscle in ALS:

As we have seen, since a very long time ALS has been considered as pathology intrinsic to the neurons, particularly motor neurons in the cortex, brainstem and spinal cord. While recent discoveries owing to the latest technologies in molecular and cellular biology have helped to disclose the secret of involvement of other cells (microglia, astrocytes and may be muscles) along with central neurons in the pathology of neurodegeneration in ALS. The importance and boundaries of a broader peripheral component are still largely underestimated and need to be elucidated. This is particularly the case of skeletal muscle, although one of the most affected tissues is considered a passive tissue undergoing the death of motor neurons. These "prejudices" however are hard to explore and explain in detail, since a detailed study of the chronology of events during the disease ALS is required.

5.1 ALS can originate from the periphery?

Despite the fact that the major focus of the majority of studies of ALS on the CNS, there are now compelling evidences to the origin of peripheral pathology. Since, the symptoms of pathology firstly appear at the motor axon resulting from damage to the distal part of the axon and at the neuromuscular junction, therefore muscle based initiation of ALS seems logical. Indeed, Fischer and colleagues found following an autopsy of a SALS patient denervation of neuromuscular junctions (NMJs) but a normal motoneuronal soma (Fischer et al., 2004). During the same study, they consistently characterized the way of motor neuron degeneration in an animal model of ALS. Following the quantitative analysis of the number of motor neurons in the spinal cord, the number of axons in the sciatic nerve as well as the degree of denervation of NMJs, they demonstrated that before the loss of motor neurons, there was a significant decrease in the number of axons in the sciatic nerve accompanied by a prominent decrease in the number of innervated NMJs. These results laid the foundation of the idea that in ALS motor neuron degeneration is a retrograde phenomenon that initiates with the destruction of the NMJ at first step.

More recently, a new concept based on various studies of neuroprotection to cure ALS has emerged that the axonal loss with NMJs denervation is the primary step that leads to the damage of cell body at the later stage. In ALS model delaying cell body degradation by protecting motor neurons treatment according to the

dying-forward hypothesis has not showed satisfactory success to delay pathology. This study figured out a new phenomenon named as “The Dying-Back” process (Dadon-Nachum et al., 2011). This hypothesis was strengthened by a series of studies. These studies show that, despite almost total rescue to the spinal motor neurons, loss of NMJs, development of muscle weakness and death of animals cannot be avoided (Gould et al., 2006; Rouaux et al., 2007; Suzuki et al., 2007; Dadon-Nachum et al., 2011; Krakora et al., 2012).

This becomes obvious from the results mentioned above that it is now doubtless the peripheral origin of neurodegeneration with NMJs as the first point of impact and disease initiation because the survival of cell bodies of motor neurons in the spinal cord through multifaceted techniques did not cure SOD1^{G93A} mice. In addition, they refer to the NMJs as a target structure for therapeutic intervention in ALS.

5.2 Hypothesis of peripheral metabolic alterations:

Another evidence of peripheral malfunction may influence the course of the disease is derived from the observation of a hypermetabolism in nearly two thirds of patients with ALS (Desport et al., 2001, 2005; Weijs, 2011). This is constant during the early condition and among these patients 80% show no change over time in their metabolic status (Bouteloup et al., 2009; Funalot et al., 2009; Vaisman et al., 2009). Such evidences of hypermetabolism in ALS patients failed to catch the attention of researchers for a long period of time due the solid and accepted concept about ALS as a neurological disease uniquely. The changes in the metabolome of ALS patients are obvious that hypothesize the involvement of metabolic pathways in neuronal differentiation and functions (Lawton et al., 2012).

In our laboratory, we have shown that mutated SOD1 mice exhibit a dysfunction of energy metabolism including a remarkable hypermetabolism in muscles (Dupuis et al., 2004) and same energetic dysfunctions have been reported in ALS patients (Ellis and Rosenfeld, 2011). Indeed, mice SOD1^{G86R} and SOD1^{G93A} also exhibit a decrease in total body weight and decreased their fat mass before the onset of motor symptoms while their food intake remains unchanged. Total energy expenditure in resting condition measured by calorimetric approach in these mice strongly demonstrated a highly noticeable hypermetabolism that was

not a result of elevated thermogenesis or hyperthyroidism (Dupuis et al., 2004). In addition, we have shown preferentially increased expression of several key enzymes involved in carbohydrates and lipid metabolism in the skeletal muscles of mSOD1 mice. Moreover, glucose uptake was found augmented in mSOD1 mice when they were injected with non-metabolizable and radiolabel analogue of glucose. All these observations suggest that the muscles being a principal energy consumer tissue are responsible for the metabolic dysfunctions observed in ALS patients and mouse models. The muscle originated hypermetabolism was then confirmed by an increase in lipid clearance, especially triglycerides, in the mutant SOD1 mouse. Indeed, following a high-fat feeding, circulating plasma lipids decreased significantly faster in mutant SOD1 mice than in their wild type counterparts. The increase in muscle-specific CD36 protein that promotes lipid uptake in muscles is an additional argument supporting a muscular origin of hypermetabolism in these mice (Fergani et al., 2007). These muscles with elevated energy requirement for normal physiological functions become hypermetabolic and lead to the NMJs denervation and ultimately motor neuron deaths. This interpretation is more close to the idea of "Dying-Back" process.

In the brain and spinal cord the metabolic changes are detectable even at every early stage of presymptomatic level in a mouse model. Niessen and his colleagues observed significant changes in N-acetyl aspartate, glutamine and γ -aminobutyric acid concentrations at the age of 34 and 75 days respectively by using proton magnetic resonance spectroscopy ($^1\text{H-MRS}$). These changes progressed along the course of disease progression (Niessen et al., 2007; Ikeda et al., 2012) and impaired glucose metabolism in a mouse model is accompanied by decreased spinal blood flow (Miyazaki et al., 2012).

There are several studies that show enhanced energy requirement by ALS patients and animal models. Ameliorative effects of energy rich diet on delaying the onset and prolonging survival to some extent have also been reported. These symptoms of hypermetabolism in other words hypolipidemia are observed even at pre-symptomatic stage of disease and they seem gender dependent (Dupuis et al., 2004; Kim et al., 2011c; Muscaritoli et al., 2012; Yang et al., 2013). In contrast, energy restriction in these mice exacerbates symptoms (Pedersen and Mattson, 1999; Hamadeh et al., 2005; Patel et al., 2010) but these ameliorative

or aggravating effects of dietary restrictions seem to be variable in different animal models of ALS (Bhattacharya et al., 2012).

Now there is no doubt in this notion that nearly all patients with the familial form of ALS manifest a constant state of hypermetabolism and enhanced energy expenditure (Desport et al., 2005; Funalot et al., 2009; Weijs, 2011; Lawton et al., 2012). Metabolic dysfunctions in particular tissues of the body that are highly affected in the neurodegeneration process for example various part of the brain, spinal cord, glial cells and muscles are being focused now (Viader et al., 2011, 2013; Cistaro et al., 2012; Fünfschilling et al., 2012).

Finally, it appears that patients with hyperlipidemia have increased survival expectancy by more than 1 year, making this report a good prognostic marker for the severity of the ALS (Dorst et al., 2011). The nutritional management of energy intake with high caloric food is being proved protective to some extent (Gonzalez de Aguilar et al., 2005; Lehericey et al., 2012). There are several ways to interrupt hypermetabolism, by interrupting enzymes responsible for mediating fatty acid oxidation, lowering the enzymatic activity of metabolism controlling hormones or by using an antagonist of metabolically active glands for example thyroids. The consequences of this elevated energy expenditure are apparently beneficial to compensate the energy requirement by some tissues like muscles. But the adverse effects of these metabolic dysfunctions are also not out of the question. This is a long and elucidation required debate that will be solved with time but hypometabolism by hypothyroidism proved effectless to survival and disease onset (Li et al., 2012).

Taking into account all these pieces of information and available data of ALS patients and animal model of ALS, it can be suggested that metabolic dysfunctions in ALS patients are muscle origin particularly. These alternations are omnipresent in all tissues with active participation in the pathogenesis of neurodegeneration. Since muscles are the tissues firstly affected during this process, it led us to focus on the role of metabolic dysfunctions in muscles as leading preliminary change in this pathology. What are the consequences of hypermetabolism or where they arise from is still under question. They may have a compensatory role against an enhanced energy requirement or causative role

to cause degeneration of neuromuscular junctions (NMJs) and finally axons and neuronal cell body death.

6. Role of Muscle in ALS pathogenesis:

The involvement of muscles in the pathogenesis of ALS is a debatable topic and needs to be elucidated by an extensive research. Although muscles are the tissues affected primarily in ALS but their role as causative tissues is still under discussion. Indeed, this tissue has been as controversial as reported by various research groups. Miller and his colleagues in 2006 excluded the involvement of muscle in SOD1 mediated pathology. They found that only muscle targeted suppression of mSOD1 expression by siRNA was not sufficient to delay motor neuron death although it ameliorated some other morphological features of muscles including muscle mass, myofibre number, and fibre diameter (Miller et al., 2006). After some years of this statement, it was reported totally opposite to the earlier stated idea. Dobrowolny and his team reported that muscle specific mSOD1 expressing mice developed the characters of muscle atrophy and reduced grip strength as observed in ubiquitously expressing mSOD1 mouse model. They observed that muscles are atrophied after being targeted by toxic effects of mSOD1 (Dobrowolny et al., 2008). This study commenced new debates of muscle involvement in pathogenesis. A very recent study has confirmed the active causative function of muscles in inducing motor neuron death. They found muscles restricted expression of mSOD1 gene in mice yielded the same phenotype of mice as observed in other ALS models (Wong and Martin, 2010).

Still it needs to interpret the role of muscle with great care before embarking on a long journey for developing therapeutic approaches. In this context, we will see what are the arguments and results of the two hypotheses stigmatized by the opposition concerning these studies.

6.1 Muscle as a passive tissue in ALS pathology:

There are only two studies that avowed the exclusion of a possible deleterious role of muscle in ALS, at least in experimental ALS models.

The very first study that keeps the muscle involvement out of this pathological process was led by Dr. Don W. Cleveland in USA. They used an approach based on intramuscular injection of a lentivirus containing siRNA directed against SOD1^{G93A} with a strong tropism for muscle tissue and not supposed to be

supported by the retrograde axonal transport to the CNS (Miller et al., 2006). In parallel, injection of AAV-siRNA SOD1 (adeno-associated virus) intramuscularly which this time will be able to use the retrograde axonal transport to infect the neuron was also tested. The results of this study, as measurement of the survival of animals and muscle strength, showed no difference between SOD1^{G93A} mice treated with lentivirus compared to their untreated counterparts, whereas mice treated with adenovirus showed improvement in muscle strength and survival. These results suggest that inhibition of the expression of mutant SOD1 in skeletal muscle does not affect the phenotype of these mice. However, despite the efforts of the authors, it seems difficult to compare the efficiencies of infection in both approaches; another limitation is the decrease of the protein, although substantial loss (approximately 50%) does not overcome the toxicity of remaining mutant SOD1.

The second exclusion has been reported in a study done in France; they used a technique based on intravenously injection of the shRNA directed against the mutated SOD1 in mice. AAVs used in this study showed an extended efficiency to a large number of cell types and to all the muscles of the animal. They found a 50% reduction in the SOD1 expression in all muscle but this reduction in muscles proved effectless against the disease onset and disease severity. The results again showed an effectless partial reduction of mSOD1 content in skeletal muscle in motor neuron death (Towne et al., 2008).

Taken together, these results suggest that the decrease in mutated SOD1 in skeletal muscle has no effect on the onset and the waning of the disease or the survival of experimental model of ALS mouse. However, most of the studies show a decrease of 50% of mutated SOD1 protein levels in skeletal muscle, it cannot be excluded that the proportion of mutant protein remaining in the muscle may be sufficient to reach threshold neurotoxicity. Moreover ubiquitously expressed mSOD1 was silenced only in muscles while it was present in other tissues. So apparently, it is a self-explanatory that in ALS not cell autonomous and only muscle restricted presence or absence of mSOD1 is not sufficient to initiate or inhibit motor neuron degeneration.

6.2 Active role of Muscle in pathology:

The hypothesis "ALS is a cell autonomous disease and only neurons are site of initiation" halted the advancement in exploring the mechanisms and therapies regarding to this fatal pathology for several years. In 2004, Fischer and his colleagues broke the ice and demonstrated that NMJs are first destabilized before the appearance of clinical symptoms both in ALS patients and mouse models (Fischer et al., 2004). Thus, studies targeting the programmed death of the neuron, although effective to prevent the loss and degeneration of neurons at the spinal level, modestly delay the death of animals (Gould et al., 2006; Dewil et al., 2007; Rouaux et al., 2007). The primary loss of these NMJs suggests that the muscle distal component of the NMJ could play an important role in the initiation of the disease.

NMJs are the distal part of motor neurons that maintain the integrity of motor neurons with muscles, the effectors. The destabilization of NMJs at the very early stage of pathology suggests a key role of this tissue in ALS pathogenesis. The loss of motor units at pre-symptomatic stage has been demonstrated by various groups (Turner et al., 2003; David et al., 2007; Hegedus et al., 2007, 2008; Gordon et al., 2010; Krakora et al., 2012). The innervation of motor unit is fibre size and type dependent and larger motor units are more vulnerable to early loss of NMJs as compared to smaller motor units. Glycolytic fibres are larger in diameter than oxidative fibre and former type of fibre is lost earlier. Functional load on the motor units accompanied with the transient switching of glycolytic fibre (fast-fatigable) into oxidative (slow) fibre is beneficial to maintain the integrity of motor units for a longer time even at the age of 90 days in the ALS mouse model. These studies demonstrate clearly that the physiological and biochemical properties of muscles contribute actively to modulate the functional and structural conditions of motor neurons. Now from the ALS pathological point of view it is still to be elucidated that whether they perform a role as a site of initiation or just as affected tissue.

The active role of muscle is self-explanatory from several evidences from various labs that show the consequences of muscles specific over expression of mSOD1. An increased SOD1 activity has been observed in the muscles in contrast to other neuronal tissues for example spinal cord and moreover this increase is independent of mutated or wild-type SOD1 gene. The gene expression increases

as the disease progresses and it is accompanied by the dysfunction of mitochondrial respiratory mechanism and can yield oxidative stress (Leclerc et al., 2001). Another clue arises from the study that demonstrates the loss of muscular mass to a noticeable extent even before the appearance of symptoms in mSOD1^{G93A} mice (Brooks et al., 2004; Marcuzzo et al., 2011). This loss in muscle mass may be a consequence of elevated mitochondrial ROS synthesis as reported in one study (Muller et al., 2007). It is postulated that this oxidative stress in muscle arises before the process of denervation and move to motor neuron cell bodies in a retrograde manner. Since by crossing ALS mouse models (SOD^{G93A} and SOD^{H46R H48Q}) with mouse carrying an antioxidant response element (ARE) reporter, the ARE system activation appears throughout the period of disease, initially in muscles (Kraft et al., 2007; Mimoto et al., 2012). We have confirmed these results by ourselves in our lab showing an early increased synthesis of superoxide radicals in the skeletal muscles of SOD1^{G86R} mice (Halter et al., 2010).

Moreover, Nogo-A, a neurite outgrowth inhibitor in CNS, expression increases in muscles both in ALS patients and mouse model. Ablation of Nogo-A in ALS mouse model prolongs the survival of motor neurons while overexpression in wild type healthy mice results in induction of denervation (Jokic et al., 2006; Teng and Tang, 2008). This idea of muscular initiated toxicity leading to NMJs dismantlement is supported by our own studies that demonstrates muscle originated hypermetabolism together with mitochondrial dysfunction is sufficient to cause neurological damage. This hypermetabolism is generated by overexpressing in tissue-specific manner, by the promoter muscle creatine kinase (MCK), uncoupling protein-1 (UCP-1) in muscle mitochondria. This protein is responsible for mitochondrial uncoupling and, consequently, a muscular hypermetabolic state that leads to an energy deficit. The metabolic dysfunction in muscles finally leads to the destabilization of JNMs, denervation and a mild axonal degeneration, a decrease in lumbar motor neurons and finally reactive astrogliosis at the spinal cord level (Dupuis et al., 2009). Similarly, Ca²⁺ signalling defects are noted in muscle mitochondria prior to the disease onset (Zhou et al., 2010). Muscles intrinsic expression of mutant SOD1 is sufficient enough to cause muscle atrophy and neuropathy suggesting the causative role of muscles in ALS pathogenesis (Dobrowolny et al., 2008; Wong and Martin, 2010).

These studies can suggest that the “The Dying-Back phenomenon” is the predominant mechanism in mSOD1 induced ALS pathology.

One possible explanation may be that muscles and neurons possess differing ability to clear the aggregates of mSOD1 by using proteasome activity. Muscles have been demonstrated to clear and deal with aggregates more efficiently (Onesto et al., 2011) and skeletal muscle satellite cells (SMSC) proliferation efficiency is compromised in SOD1^{G93A} mouse model of ALS (Manzano et al., 2013). This shows that once muscles reach their threshold to clear and modify mSOD1 aggregates along with their repairing capacity, they suffer from the atrophy and toxic effects. This toxicity migrates from muscles to other cells such as innervating motor neurons through a mechanism yet not disclosed. While inhibiting synthesis of ROS in muscles by injecting recombinant human heat shock protein 70 (rhHsp-70) into the muscles delayed the NMJs degeneration and ameliorated life span of mice. The injected exogenous rhHsp70 localizes to the muscles and not to motor neurons of spinal cord or brain (Gifondorwa et al., 2012). Muscle-Specific Kinase (MuSK), a receptor tyrosine kinase, induces NMJs formation and is required for NMJs integrity. Promoting nerve terminal attachment by inducing retrograde signalling of MuSK can prevent NMJs dismantlement in pathological conditions. Mice bearing mSOD1^{G93A} were crossed with transgenic mice that express MuSK and histological and behavioural assays were used to assess motor innervation and behaviour. A 3-fold increase in MuSK level delayed the onset of disease, reduced muscle denervation, improved motor function for more than one month without changing survival.

Muscles based therapeutics is being emphasized now a days (Shefner, 2009; Dupuis and Echaniz-Laguna, 2010). Glial cell derived neurotrophic factor (GDNF) is a well-established growth factor with protective and ameliorating effect on suffering neurons. In 2007, one group reported that muscles derived GDNF have more profound protective actions on motor neurons in ALS model mice as compared to astrocyte derived GDNF (Li et al., 2007). Next to this primary study, there are many evidences proving this idea of muscle being the most suitable and effective site for therapeutic intervention. In this way GDNF and Insulin like Growth Factor-1 (IGF-1) have been tested to treat ALS mice models. The expression of muscle and non-astrocytic GDNF by transgenesis or by injection of human mesenchymal stem cells expressing GDNF in muscle (hMSC-GDNF)

significantly improves survival, number of neurons, strength and condition of mutated SOD1 rats (Suzuki et al., 2008). Meanwhile, overexpression of IGF-1 in muscle of SOD1^{G93A} mouse allows NMJs stabilization, reduced CNS inflammation, and can delay the onset of symptoms along with increased survival of mutant mice (Dobrowolny et al., 2005). These results have been confirmed by showing that the expression of a plasmid containing Mechano-growth factor (MGF) a splice variant of IGF-1, in muscles has offered the same beneficial effects in mSOD1 model (Riddoch-Contreras et al., 2009).

Taken together, all these results suggest that skeletal muscle is the site of disease initiation and is a primary target of toxicity induced by the mutant SOD1 gain of function, indicating the muscle as a target to study the disease mechanisms and remedies for ALS.

Based on the present evidences, one can postulate the alterations such as metabolic dysfunctions in neurons or in their periphery can serve as markers of disease initiation. Metabolic dysfunction or energy imbalanced state is observed in most of ALS patients and mouse models that provoke the temptations in the scientific community to focus on this aspect for further exploration. We have already observed the modified expression of genes that modulate the lipid oxidation in the ALS mouse model. Stearoyl-CoA desaturase-1 (SCD1) is one of these factors that catalyse the synthesis of MUFAs from SFAs and results in elevated lipid peroxidation. Our gene profiling study suggested an active involvement of this gene in ALS pathogenesis.

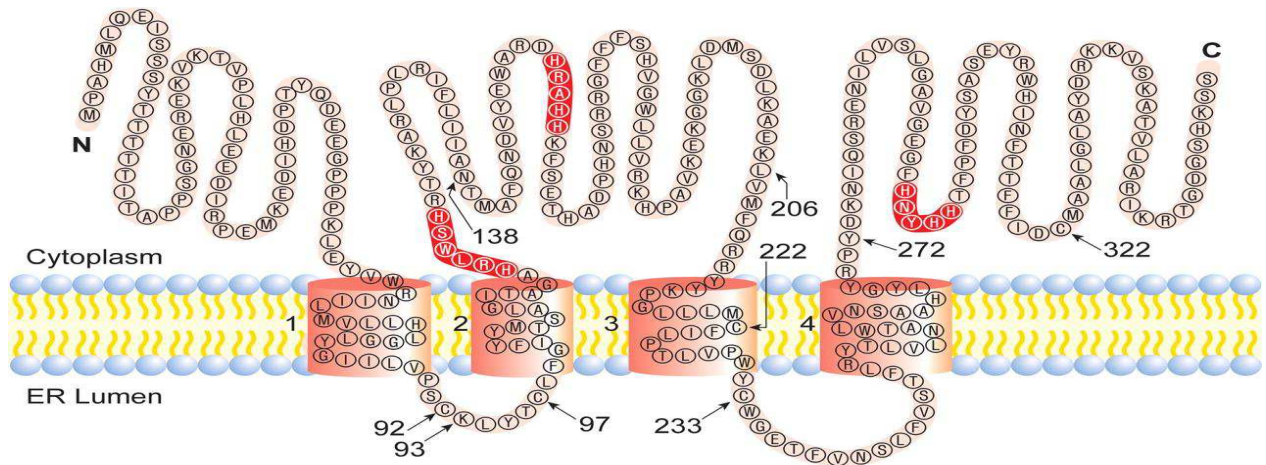
A number of diseases reported till date arises from the alterations in expression of SCD1 and its products MUFAs to SFA ratios. The disorders in which SCD1 expression or activity alterations can be implicated are; lipotoxicity (Paumen et al., 1997; Cnop et al., 2001; Lee et al., 2010; Virtue and Vidal-Puig, 2010), obesity (Enser, 1975; Smith et al., 1999; Warensjö et al., 2006; Hodson et al., 2010; García-Serrano et al., 2011; Gong et al., 2011; Vessby et al., 2012), liver steatosis (Stefan et al., 2008; Kotronen et al., 2009; Li et al., 2009b; Petersson et al., 2010), cardiovascular disorders (Peter et al., 2008; Warensjö et al., 2008; Dobrzyn et al., 2010a, 2012), pancreas related diseases (Busch et al., 2005; Thörn et al., 2010), insulin resistance (Warensjö et al., 2005, 2006, 2008; Sjögren et al., 2008; Stefan et al., 2008; Roberts et al., 2009; Vessby et al., 2012), inflammation (Baer et al., 2004; Chen et al., 2008; Petersson et al., 2008, 2009; Calder, 2010), cancer (Moore et al., 2005; Scaglia and Igal, 2005, 2008; Scaglia et al., 2005; Hess et al., 2010; Igal, 2010; Chajès et al., 2011; Roongta et al., 2011) and bone diseases (Takeda et al., 2002; Akune et al., 2004; Eleftheriou et al., 2005; Melhus et al., 2008). The modulation of SCD1 expression leading to the altered MUFAs/SFAs ratio may have ameliorating or deleterious effects on disease progression or initiation in the disorders mentioned above. Administration of such kind of metabolites may be used as a therapeutic approach to cure health and life threatening maladies and it asks for constant huge struggles in this regard. Since muscles are the key tissues that are affected by neurodegenerative disorders such as ALS a primary focus of our study and our laboratory, a brief influence of SCD1 expression or activity aberration on muscle related pathological conditions is discussed below. Here we briefly

introduce SCD1 functions, isoforms, factors affecting its expression and involvement in various pathologies.

7. Stearoyl-CoA Desaturase-1 (SCD1):

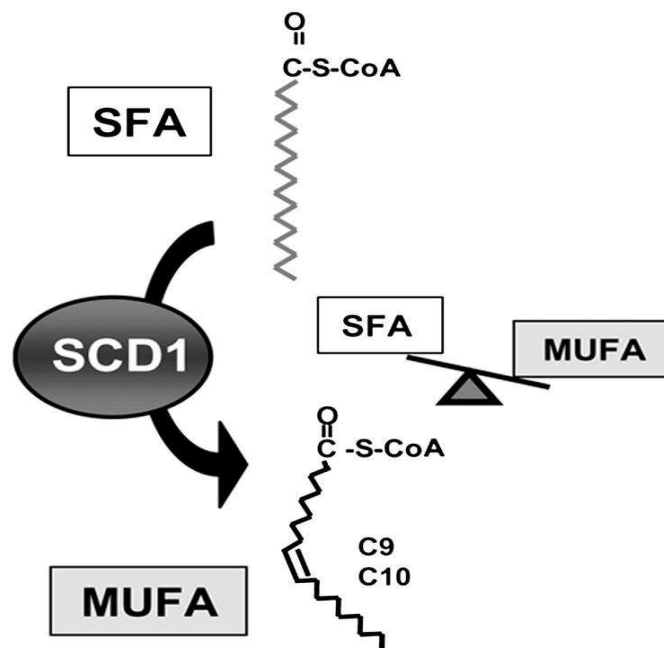
Stearoyl-CoA desaturase-1 (SCD1), a delta-9 desaturase is an enzyme that plays an important role in metabolism by controlling the synthesis of Monounsaturated Fatty Acids (MUFAs) from Saturated Fatty Acids (SFAs) (Fig. 4b). It participates in the synthesis of MUFAs from saturated fatty acyl precursors by a three-component enzyme system that involves flavoprotein-NADH-dependent cytochrome b5 reductase, cytochrome b5, and SCD1. SCD1 also regulates the synthesis of triglycerides and very low density lipoproteins (VLDL) in the liver. It is a short-lived protein, bound to the endoplasmic reticulum (ER) membrane in eukaryotic cells and is associated with electron transport chain in liver microsomes. Its expression is under multiple factors, for example transcriptional factors and protein degradation components (Heinemann and Ozols, 2003). It is suggested that membrane localized desaturases consist of four membrane spanning domains with C and N termini that are towards the cytosolic side. Particularly mouse SCD1 protein consists of 355-amino acids, contains four transmembrane domains (TMDs) and three loops connecting the membrane-spanning domains. The four transmembrane cytosolic directed domains are separated by two very short hydrophilic loops in the endoplasmic reticulum (ER) lumen along with a single cytosolic large hydrophilic loop while cysteines are not essential for its catalytic activity (Fig. 4a) (Gene et al., 1990; Man et al., 2006b; Hodson and Fielding, 2013).

Fig. 4a: Proposed model of mouse Stearoyl-CoA desaturase-1



This model of SCD1 shows that four transmembrane domains 1-4 and the N and C termini that are oriented towards the cytosolic part. The two ER luminal loops are relatively smaller as compared to the cytosolic loop that possesses two of the three conserved histidine motifs. The highlighted residues are conserved histidine regions which are essential for catalytic functions. The five cysteines are located at 92, 97, 222, 233, and 322 residues in SCD1 (Man et al., 2006b).

Fig. 4b: SCD1 enzymatic activity



Modified from (Igal, 2010)

SCDs or delta-9 desaturase genes are conserved from higher organisms to unicellular fungi and there is great variability in SCDs gene complement of vertebrates. There are four isoforms of murine SCDs; SCD1, SCD2, SCD3 and SCD4 in a 200 kb span of chromosome 19. SCD1 is well characterized and has been extensively studied in the last few decades. In human, two isoforms SCD1 and SCD5 have been identified so far. Human SCD1 shows 85% homology with murine four SCDs isoforms while SCD5 is unique in human (Sampath and Ntambi, 2006; Evans et al., 2008; Castro et al., 2011).

In human SCD1 is located on chromosome 10 and SCD5 is found on chromosome 4 (Wang et al., 2005c). SCD1 and SCD2 in mouse and SCD in rat have single sequence for a functional polyadenylation. One study has demonstrated varying transcript of SCD in various human tissues and suggested the presence of more polyadenylation sites in SCD for alternative usage leading to tissue specific activity (Zhang et al., 1999).

The characterization and isolation of SCD have always been a difficult task because of its fragile nature and membrane association. To date only one mammalian delta-9 desaturase enzyme from rat liver has been successfully purified from microsomes by using detergent extraction method. Pure SCD migrates as a single polypeptide on SDS-PAGE with 37 kDa approximate molecular weight (Heinemann and Ozols, 2003; Hodson and Fielding, 2013). The stability of SCD enzyme is highly fragile, even at -80°C highly purified SCD can be stored and the presence of detergent can enhance degradation (Hodson and Fielding, 2013). It has also been shown that the decline in desaturase activity in microsomes is a result of protein disappearance or degradation. Various proteases such as plasminogen-like protein and microsomal endopeptidase that result SCD degradation have been reported (Ozols, 1997; Heinemann et al., 2003a, 2003b)

The proteins that are found in ER membrane do not last for a longer period of time but there is a continuous synthesis and degradation with an average half life of about 2 days. No doubt the protein degradation rate varies for various proteins and for different species of their origin. The half life of hepatic SCD is very short that ranges from 3 to 5 hours only but the phenomenon of this quick degradation is to be elucidated yet. The ratio of SFAs to UFAs is under the strict

control of SCD stability; synthesis and degradation (Ozols, 1997; Heinemann and Ozols, 2003; Heinemann et al., 2003a).

SCD1 desaturation activity is highly specific to its substrates. The most common substrates for SCD1 are acyl derivatives of 18:0 and 16:0, the most abundant component of all lipid fractions in nearly all living beings. In one study the tendency of SCD1 substrates specification has been established by using partially purified extracted SCD1 enzyme from rat liver microsomes. It shows the specification of activity in the following descending order 18:0 > 16:0 > 14:0 > 15:0 (Jeffcoat et al., 1977). When substrates are free CoA, free fatty acids or short chain acyl-CoA, this enzyme shows no inclination to bind with these substrates (Enoch et al., 1976). The various isoforms of SCDs in mouse are not only tissues specific in expression but also demonstrates the specificity in substrate selection. It has been found the SCD1, SCD2 and SCD4 desaturase more preferably 18:0 as compared to 16:0 but show some activity to 16:0 while SCD3 is more specific in desaturating 16:0 and is effectless to 18:0 (Miyazaki et al., 2006).

In an *in vivo* study done in human, desaturation of 16:0 and 18:0 has been demonstrated by using fatty acid stable isotope tracers; [U-¹³C] stearic acid (18:0) and [U-¹³C] palmitic acid (16:0). Conversion of 18:0 to 18:1 was 14% and conversion of 16:0 to 16:1 was 2% in plasma over a period of 7 days (Rhee et al., 1997; Hodson and Fielding, 2013).

8. Tissue specific expression of SCD in human and rodents:

Besides the fact that all delta-9 desaturases in most of the species share a fear homology but they show a tissue specific expression. Due to the varying number of isoforms in human and rodents, it is self-explanatory regarding to the functional overlapping with 4 isoforms in rodents and only 2 in human.

8.1 Man:

The expression of SCD1 is more abundant in liver and adipose tissues. This expression is similar to its orthologues in mice but while comparing the quantity of expression, it is lesser than that of SCD1 in murine liver (Wang et al., 2006). Human SCD5 was first reported in 2005 with expression restricted to the brain and pancreas. It was found that already reported human gene ACOD4 encodes a

functional protein resembling to delta-9 desaturase and was named as SCD5 (Wang et al., 2005c; Lengi and Corl, 2008).

8.2 Rodents:

Under normal dietary conditions in adult mice the SCD1 mRNA is highly expressed in lipogenic tissues (white adipose tissue, brown adipose tissue), harderian gland, Meibomian gland, preputial gland and liver. On provision of high-carbohydrate diet, SCD1 expression is dramatically induced in liver and heart (Ntambi, 1999; Paton and Ntambi, 2009).

SCD2 expression is found predominantly high in brain in young pups during the period of myelinisation. It also shows high carbohydrate food mediated some expression in kidney, heart, lung and spleen that is. The expression of SCD2 is also detected in adipose tissues and eyelid along with SCD1 expression (Ntambi, 1999; Miyazaki et al., 2005).

SCD3 is restricted in its expression to the sebocytes of sebaceous glands in the skin, preputial and harderian glands of mouse skin. Its expression is gender dependent; higher expression in male mouse skin and lower in female mouse skin (Zheng et al., 2001; Paton and Ntambi, 2009; Hodson and Fielding, 2013). SCD4 is found only in the heart (Miyazaki et al., 2003).

Under normal dietary conditions, rat SCD1 and SCD2 expression is detectable in liver, brain, testis and kidney. SCD2 expresses in the brain and its expression is induced in response to high carbohydrate diet in other tissues for example lung, spleen, kidney and adipose tissues (Kaestner et al., 1989; Mihara, 1990; Hodson and Fielding, 2013).

9. The role of SCD in lipid metabolism:

9.1 De novo lipogenesis:

De novo lipogenesis (DNL) is supposed not to increase fat stores in the body but this cellular process seems to serve a key role to regulate elongation and desaturation of SFAs in adipocytes. DNL and SCDs activity are strongly correlated to maintain a limited and required level of TAG, MUFAs and cholesterol according to the demand (Fig. 5). SCDs catalyse the synthesis of MUFAs from its substrate SFAs that are synthesized endogenously or provided exogenously by diet (Yee et al., 2008; Collins et al., 2010, 2011). Moreover, fatty acids

elongation and desaturation function parallel to the up-regulation of genes that mediate the DNL; this suggests the presence of a coordinated control of gene expression. Enhanced level of DNL in liver parallel to the desaturation of fatty acids has been reported in response to a high carbohydrate diet (Aarsland and Wolfe, 1998; Chong et al., 2008).

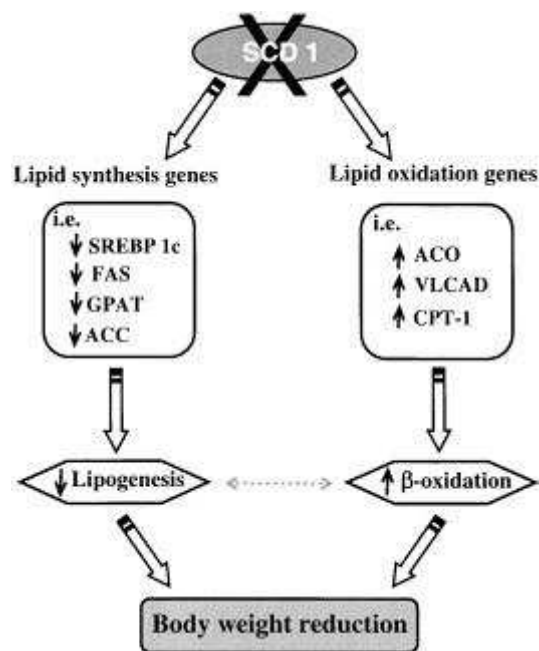
9.2 Triacylglycerol (TAG):

Triacylglycerols (TAG) are esters of glycerol and fatty acids and constitute a major portion of human skin oils. It is reported that SCD1 gene expression is necessary for the synthesis of TAG because in mice with targeted disruption of SCD1 gene, very low level of VLDL was observed and exogenously provided MUFAs could not restore the endogenously synthesized MUFAs necessary for TAG. These studies figured out the proposal that de novo synthesis of MUFA by SCD1 is essential for TAG synthesis (Miyazaki et al., 2000, 2001a). However, this proposal cannot be generalized for the human being where provision of exogenous dietary MUFAs is sufficient enough to meet the required quantity of MUFAs for TAG synthesis and as a result endogenous synthesis of MUFAs can be compromised.

In the synthesis of TAG, the addition of a third Acyl chain to DAG finalizes the process of TAG synthesis. This process is catalysed by Acyl-Coenzyme A: diacylglycerol acyltransferase (DGAT), a membrane bound enzyme (Weiss et al., 1960; Hodson and Fielding, 2013). DGAT1 and DGAT2, the two DGAT genes have been reported so far and DGAT2 is localized mainly to the ER (Suzuki et al., 2005). In an *in vitro* study, SCD1 and DGAT were observed closely located in ER. The cells with higher expression of both SCD1 and DGAT2 showed greater accumulation of TAG than cells with overexpression of either of these genes. These results lead to the conclusion that exogenously provided stearate and palmitate in diet or endogenously de novo synthesized fatty acids are desaturated by SCD1 and are channelled to DGAT for the final step of TAG production in ER. This intimate association seems to ameliorate the efficiency of the process of TAG production (Man et al., 2006a). Very recent studies by using stable-isotope have elucidated that DGAT2 functions on the upstream of DGAT1. DGAT1 mediates the esterification of dietary fatty acids while DGAT2 takes part in the synthesis of TAG by using desaturated fatty acids (Qi et al., 2012; Wurie et al., 2012).

Therefore, an indirect role of SCD by regulating fatty acid synthesis is suggested for the synthesis of TAG rather than a direct role (Jeffcoat et al., 1979). It can be summarized that SCD1 indirectly affects the secretion of lipoproteins by handling the amount and types of fatty acids for TAG production. But this notion is yet to be elucidated by further work (Hodson and Fielding, 2013).

Fig. 5: SCD1 in lipogenesis and lipolysis



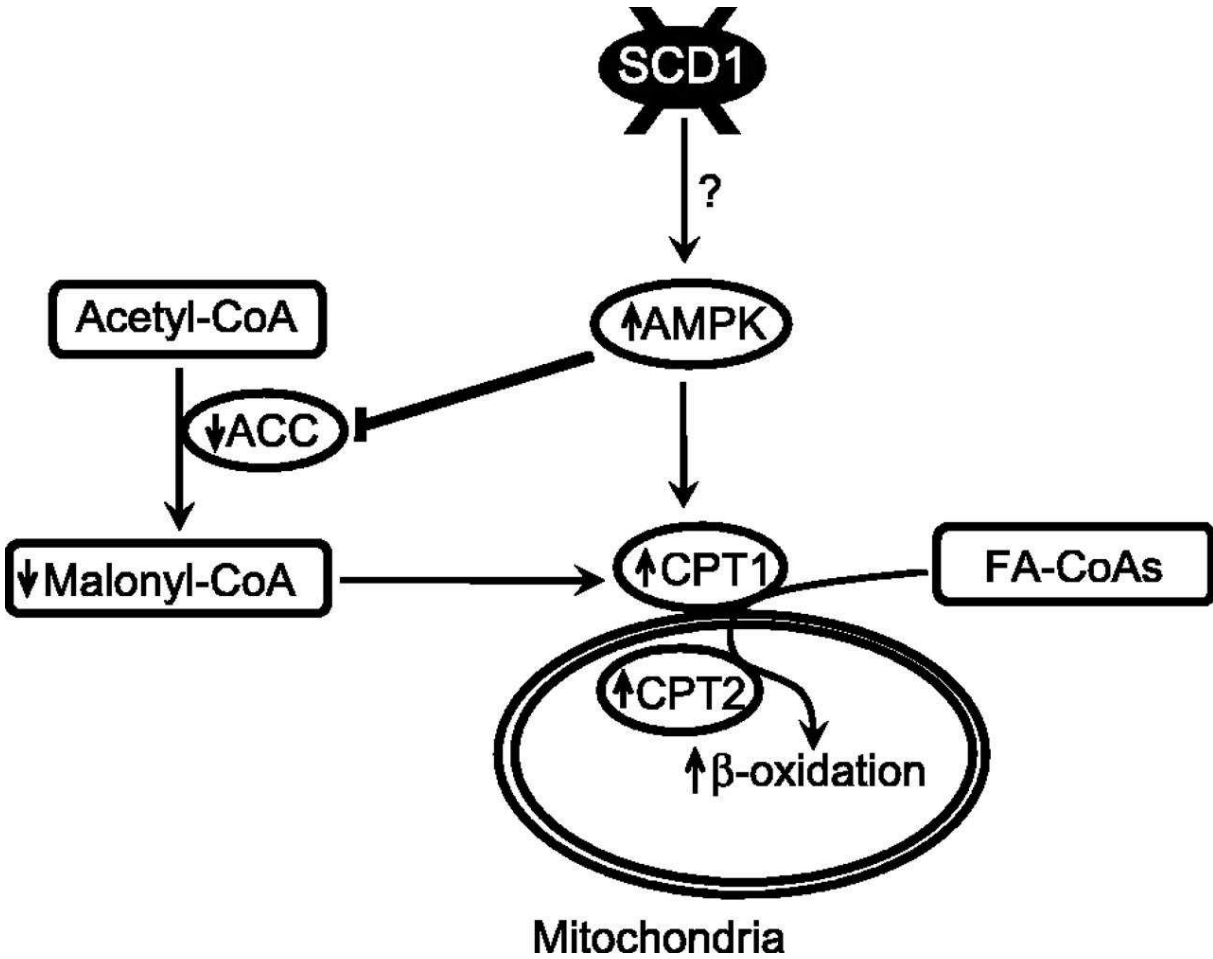
(Dobrzyn and Ntambi, 2004)

9.3 Fatty acid oxidation and thermogenesis:

Although SCD1 plays an essential role in lipogenesis, but SCD1 is also implicated in enhancing lipolysis and thermogenesis. A unique feature of SCD1-deficient mouse model is elevated fatty acid oxidation. The increased fatty acid oxidation greatly provides protection from diet-induced obesity (Cohen et al., 2002; Ntambi et al., 2002; Dobrzyn et al., 2004, 2005a; Lee et al., 2004; Sampath et al., 2007). Following a four-hour fasting, level of β -hydroxybutyrate was significantly higher in *Scd1*^{-/-} mice as compared to control group, indicating enhanced fatty acid oxidation in *Scd1*^{-/-} mice (Ntambi et al., 2002). *Scd1*^{-/-} mice show increased fatty acid oxidation in liver, brown adipose tissue (BAT) and skeletal muscles (Dobrzyn et al., 2004, 2005a; Lee et al., 2004). This fatty acid oxidation is mediated by AMPK induction which results in phosphorylation and inactivation of ACC, and a consequent induction of CPT-1 enzyme that transports fatty acids into the mitochondria for β -oxidation (Dobrzyn et al., 2004; Kim et al., 2011a). In addition, malonyl-CoA a product of ACC, acts as a repressor of CPT-1. Reduction in *Acc* gene expression in *Scd1*^{-/-} mice (Miyazaki et al., 2001a, 2004a, 2007; Sampath et al., 2007) likely plays an important role in upregulating fatty acid oxidation. Moreover, the genes that stimulate fatty acid oxidation such as acyl CoA oxidase, *Cpt-1* and very long-chain acyl CoA dehydrogenase also show increased expression in *Scd1*^{-/-} mice (Ntambi et al., 2002).

Nuclear receptor PPAR α is an important player in transcriptional regulation of fatty acid oxidation (Sampath and Ntambi, 2005), but it does not appear to be essential for the induction of oxidation due to SCD1 deficiency. The removal of SCD1 in PPAR α -null mice does not offset the effects of SCD1 deletion on fatty acid oxidation (Miyazaki et al., 2004b). In addition, thermogenesis is also significantly enhanced along with increased fatty acid oxidation in *Scd1*^{-/-} mice. Activation of the 3-adrenergic receptor signalling pathway in BAT in *Scd1*^{-/-} mice induces the expression of peroxisome proliferator-activated receptor-gamma coactivator-1 α (PGC1- α) and uncoupling protein-1 (UCP-1). This process increases the rate of basal thermogenesis and ultimately augmented whole body energy expenditure (Fig. 6) (Lee et al., 2004; Sampath and Ntambi, 2011).

Fig. 6: β -Oxidation by SCD1 deletion



(Dobrzyn et al., 2004)

10. Regulation of SCDs expression:

Along with transcription regulation of SCD1 expression, SCD1 protein stability is also among the determinant factors that control its expression (Fig. 7). SCD1 protein itself is a short lived protein with a half-life of 3 hours approximately but ubiquitin-proteasome-dependent ERAD system can also affect the constitutive SCD1 degradation through ATPase p97 protein recruitment (Kato et al., 2006). The expression level of ATPase p97 mRNA does not vary in subcutaneous and visceral adipose tissues in both obese and control objects. This expression seems to be tissue specific with higher expression in visceral and lower in subcutaneous adipose tissues (García-Serrano et al., 2011). Other factors that affect SCD1 expression are discussed in detail in the following sections.

10.1 SREBP-1C:

The expression of genes involved in lipogenesis is under the tight transcriptional control of genes containing sterol responsive element (SRE). The sterol responsive element binding proteins (SREBPs) are found in various tissues associated with ER membranes. SREBP-1c mostly increases the synthesis of fatty acids by enhancing the transcription of lipogenic genes; for example SCD1, while SREBP-2 influences the biosynthesis of cholesterol through inducing gene expression (Ntambi, 1999; Kim et al., 2002a; Heinemann and Ozols, 2003). SREBP-1c^{-/-} mice show a decreased expression of hepatic SCD1 mRNA whilst mice expressing a constitutively active form have increased expression. Mice over expressing constitutively active form of SREBP-1c exhibit an enhanced level of SCD1 mRNA (Shimomura et al., 1998; Liang et al., 2002).

10.2 Insulin:

There is a set of strong evidences that suggests the positive control of SCD1 activity by Insulin. Hepatocytes when incubated with insulin, displayed an elevated SCD1 activity and withdraw of insulin caused a decline in SCD1 activity by 80%. These results suggested that insulin affected SCD1 expression through various channels for example; it seems necessary for the synthesis of enzyme, maintains its activity and protects its protein from degradation (Jeffcoat et al., 1979). A contemporary study also showed similar outputs; in a rat model of streptozotocin-induced diabetes, hepatic SCD1 activity was declined 3.7 times and on receiving insulin treatment this activity was not only restored but was

observed enhanced up to 7 times (Prasad and Joshi, 1979). However, along with this direct transcriptional control over SCD1 expression, insulin mediates SCD1 expression by controlling SREBP-1c expression (Horton et al., 1998; Shimomura et al., 1998, 1999a, 1999b, 2000). Dibutyryl-cAMP and theophylline (cAMP) shows a negative control on insulin mediated expression of SCD1. In a study, insulin mediated over expression of SCD1 was reduced by 75% when these animals were offered cAMP confirming a negative control of the hepatic cAMP on the expression of SCD1 *in vivo* (Waters and Ntambis, 1994).

10.3 Leptin:

Leptin is synthesized by adipose tissues and it regulates feedback of nutritional status to the hypothalamus that is a food control centre in the brain. Mostly it is secreted by white adipose tissues but not exclusively. The level of leptin is positively maintained by adipose tissue size; larger the tissues more the leptin abundance and vice versa. Generally leptin inhibits the food intake and maintains energy expenditure and food intake equilibrium. Leptin maintains energy homeostasis and leptin level modifications have been reported to affect many other systems, energy balance, metabolism, neuroendocrine responses to nutritional status, reproductive system, bone formation and the immune system (Zhang et al., 1994; Friedman, 2000). Administration of leptin in leptin-deficient (ob/ob) obese mice and human resulted depletion of adipose tissues and removal of lipids in the liver and other peripheral tissues (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Farooqi et al., 1999, 2002). Mostly murine models have been used to investigate the influence of leptin on SCD1 expression (Cohen et al., 2002; Biddinger et al., 2006; Flowers et al., 2007; Miyazaki et al., 2009). Hepatic SCD1 gene expression alteration by leptin treatment was firstly demonstrated in 2002. In a microarray analysis, the investigator identified hepatic SCD1 expression was decreased by leptin treatment and this was classified in the highest rank in that study. Leptin treatment governed many characteristics of this gene such as, repressed expression, decreased enzymatic activity, and low abundance hepatic MUFAs such as 16:1 n-7 and 18:1 n-9 in treated mice as compared to untreated control mice (Cohen et al., 2002). This leptin mediated suppression of SCD1 expression is independent of SREBP-1c and insulin (Biddinger et al., 2006; Miyazaki et al., 2009; Hodson and Fielding, 2013).

10.4 Sex differences:

Sexual dimorphism related to body weight, size, lipid oxidation rate, fats handling capacity, fat storage and other features in human has been documented by many studies. Based on such information, a differential expression of genes such as SCD1 involved in lipid metabolism in liver, white adipose tissues (WAT) and muscles is suggested. A number of reports have strengthened this idea with these findings; female rats showed a better lipid handling capacity due to higher storage in adipose tissues and elevated oxidation rate of fatty acids in effector tissues (muscles) as compared to opposite gender (Blaak, 2001; Priego et al., 2008). Another investigation came out with elucidation of sexual dimorphism of SCD1 expression in mice. The study showed a higher expression of SCD1 mRNA in female as compared to male mice (Lee et al., 1996) and in human (Warensjö et al., 2006). Experiments done with rats and mice have shown the elevated expression of hepatic SCD1 mRNA in female irrespective of the strain. The female showed higher quantity of SCD1 desaturated fatty acids palmitoleate and oleate in the total liver lipids and in plasma CE ratio (Lee et al., 1996; Warensjö et al., 2006; Priego et al., 2008).

In female fats are mostly deposited in gluteofemoral adipose tissues while in man this deposition is mostly in visceral portion. In both tissues of female subjects SCD1 mRNA expression was higher than that in male adipose tissues (Blaak, 2001; Pinnick et al., 2012). All *in vivo* studies on animal models have figured out that fat deposition and SCD1 activity increased are always parallel. It has been hypothesized that the sexual dimorphism observed in the accumulation of fats in different part of the body may be elucidated by the variable SCD1 mRNA expression level. One possible explanation for the mechanistic phenomenon in elevated SCD1 expression in female can be supposed that particular sex hormones alter this expression. Investigation of variations in the MUFAs ratios and SCD1 expression in female before and after menopause period can also help to unravel the facts (Enser, 1979; Giltay et al., 2004; Hodson and Fielding, 2013).

10.5 Dietary influences:

10.5.1 Carbohydrates:

Dietary carbohydrate effects on the expression of SCD1 and its activity have been documented by various studies. Most of the studies have figured out that increased carbohydrate contents decrease the fat intake. A diet rich in carbohydrate contents has been reported as a strong provoker of DNL in human liver (Aarsland and Wolfe, 1998; Chong et al., 2008). This enhanced DNL is preceded by hypertriglyceridemia and insulin resistance as reviewed by a number of reviewers (Strittmatter et al., 1974; Fried and Rao, 2003; Chong et al., 2007; Parks, 2007; Stanhope and Havel, 2008). Studies done on animal models have clearly demonstrated the induction of SCD1 expression and activity in liver by increased dietary contents of carbohydrates (Strittmatter et al., 1974; Thiede and Strittmatter, 1985; Ntambi, 1992). The diet rich in carbohydrate contents also provokes the increased expression of SCD1 in adipose tissues while its expression in other tissues including testis, brain and kidneys remains unchanged (Mihara, 1990).

The alteration in expression of SCD1 by carbohydrate diet is suggested to be mediated at transcriptional level. Animals on a carbohydrates rich and fat free diet following a long fasting, yielded 2-fold increase in 6 hours and 45-fold increase in 36 hours in SCD1 mRNA expression. A persistent increase in SCD1 expression was reported until animals were nourished on high-carbohydrate contents diet while switching to normal chow diet resulted in a sharp decrease in SCD1 mRNA expression (Ntambi, 1992). It has been assumed that the response of SCD1 to refeeding reflects the reciprocal effects of glucose and insulin which would enhance after feeding with a high-carbohydrate diet (Heinemann and Ozols, 2003). Chong et al., 2008 has investigated the changes in the activity and expression of SCD1 in liver and adipose tissues in response to short-term alteration in total carbohydrate intake. The Subjects were nourished on a carbohydrate rich diet (75% total energy) or fat (40% total energy) for a period of 3 days before the day of postprandial study and switching to the alternate diet after a six week washout period. It was noted that DNL and SCD1 expression were elevated in response to high-carbohydrate diet (Chong et al., 2008). Taken together, it can be concluded that carbohydrate rich diet enhances SCD1 expression.

10.5.2 Fructose:

Fructose is a monosaccharide and is normally found as a component of sucrose (table sugar) that is a disaccharide in nature.

Mostly murine and rat models have been brought under investigation to explore the effect of dietary fructose on SCD1 expression and its activity (Waters and Ntambis, 1994; Miyazaki et al., 2004a; Koo et al., 2009; Kim et al., 2011b). All these studies have borne the results that a diet rich in fructose increases SCD1 expression in mice (Waters and Ntambis, 1994; Miyazaki et al., 2004a; Kim et al., 2011b) and in rat liver (Koo et al., 2009). While fructose feeding in SCD1 ko mice, resulted a remarkable decline in plasma TG abundance up to 54% as compared to wild type littermates. When these SCD1 deficient mice were fed a fructose rich diet supplemented with triolein (18:1, Oleate), they showed a 4.8-fold increased level of TG in liver and normalized plasma TG abundance. Interestingly, the fructose rich diet supplemented with SFA such as tristearin (18:0) or tripalmitin (16:0) was found effectless against the observed enhancement of TG in liver and plasma of SCD1 ko mice (Miyazaki et al., 2004a). This study provoked authors to conclude that a product of SCD1 oleate (18:1) is necessary for lipogenesis mediated by dietary fructose and effect of palmitoleate (16:1) on TG concentration in liver and plasma remains to be elucidated by further studies.

TR4 is a member of the nuclear receptor superfamily that plays in role in lipid metabolism and SCD1 expression in metformin→AMPK→TR4→SCD1→insulin sensitivity manner. TR4 knockout mice show a suppression of SCD1 mRNA expression in liver. The author found that when TR4 knockout mice were nourished on fructose rich diet, they exhibited an induction of 60% in hepatic SCD1 mRNA level as compared to control group. So it was suggested by the author that fructose mediates SCD1 expression in a TR4-independent way (Kim et al., 2011b). Fructose provision enhances the level of hepatic DNL in human, it has been documented by one group but data regarding to the SCD1 mediation by fructose in human beings is very limited (Faeh et al., 2005). Another investigation revealed that SCD1 mRNA expression augmented in muscle of healthy men after eating fructose rich diet (Lê et al., 2008).

Taken together, these pieces of information demonstrate that fructose in one way or other regulates the expression of SCD1 in a positive manner.

10.5.3 Saturated fatty acids (SFAs):

Saturated fatty acids are fatty acids with no double bond and have a high melting temperature. An investigation was done on mice and hepatocytes. Hepatocytes incubated with SFAs showed increased expression of SCD1. The mice fed SFAs rich diet had increased hepatic SCD1 expression as compared to the mice fed a normal chow diet (Li et al., 2009b). In recent studies, the effects of a diet containing tristearin (18:0) have compared with the diet containing triolein (18:1, Oleate) by using various models (Miyazaki et al., 2000; Sampath et al., 2007; Dobrzyn et al., 2012). When rats were fed on a diet rich in tristearin (18:0), they showed a notable higher level in the cardiac SCD1 mRNA expression than that in animals fed on a normal chow diet (Dobrzyn et al., 2012). The wild type mice showed the same trend in the expression of the SCD1 level after being nourished on tristearin rich diet. The mice on tristearin containing diet exhibited a higher expression of hepatic SCD1 mRNA level than the mice on triolein containing diet (Sampath et al., 2007).

The studies relating to SCD1 expression in human in response to such diet are very rare. A very limited number of investigations have been performed to find out the relationship of SFAs and SCD1 expression. Most of the studies have been with murine models and some with cellular models to explore the effects of SFAs on SCDs expression. Mostly the effect of saturated fatty acids on SCDs gene family is calculated by comparing the products of these genes with other fatty acids, so the interpretation of results is not so straight forwards rather it is challenging. It becomes more challenging when the comparison is made between SFAs and PUFAs.

10.5.4 Monounsaturated fatty acids (MUFAs):

In an *in vitro* study by using β -cells, It was noted that MIN6 cells incubated with MUFAs namely palmitoleate (16:1) or oleat (18:1) for a period of 48 hours showed a significant decrease in SCD1 and SCD2 expression (Thorn and Bergsten, 2010). When tristearin and triolein feeding effects were investigated, triolein (18:1, Oleate) feeding did not modify the expression of hepatic SCD1 mRNA expression in mice (Sampath et al., 2007). In a recent

study, the effect of MUFAs namely oleate on SCD1 gene expression was explored. FAO hepatoma cells were treated with oleate for 48h and it was observed no difference in SREBP-1c, carbohydrate response element binding protein (ChREBP) and fatty acid synthase (FAS) expression as compared to control cells, although the expression of Acetyl CoA Carboxylase (ACC) was found to increase significantly. The protein level of SREBP-1c and ACC were found comparable with control cells. These data can be interpreted in this way; since oleate showed no effect on SREBP-1c and FAS expression, it would also have no effect on SCD1 expression (Kaur et al., 2011). Though data are very limited but it can be speculated that MUFAs may modestly affect the expression of SCD1.

10.5.5 Polyunsaturated fatty acids (PUFAs):

Since very long, it has been well established that dietary linoleic acid (18:2 n-6) when provided as safflower oil, suppresses the synthesis and half-life of fatty acid synthase (FAS) as compared to the diet voided of fats (Flick et al., 1977). Therefore, it provoked to suppose that SCD1 expression would also be affected in the same manner. The young rats having a diet rich in linoleate (60% w/w) showed a sharp decrease in the activity of SCD1 in liver (Jeffcoat and James, 1978).

The suppression of SCD1 expression by linoleic acids was very strong because it was reported that 1g of dietary linoleic acid could nullify the inductive effects of 18g sucrose in rat. If applies to human, 3-4g of linoleic acid ingestion per day would be enough to remove the inductive effects of 60g sugar on expression of SCD1 in liver (Jeffcoat and James, 1977). The effects of PUFAs were investigated by feeding mice on a fat free diet supplemented with TGs and PUFAs. It was reported that when diet was supplemented with any of PUFAs trilinolenin (linolenic acid, 18:3n-3), trilinolein (linoleic acid, 18:2n-6) and triarachidonin (arachidonic acid, 20:4n-6), it decreased the induction of hepatic SCD1. It was noted that suppression effect of PUFAs was apparently dependent on carbon and double bond number as triarachidonin exhibited very striking effects on SCD1 mRNA suppression (Ntambi, 1992). Insulin stimulated expression of SCD1 mRNA blunted on exposure of linoleic acid and arachidonic acid in an in vitro study of H2.35 cell line (Ntambi et al., 1996). Similarly, INS-1 β cells showed a decreased expression of SCD1 when their medium was supplemented with arachidonic acid (Ramanadham et al., 2002).

A high PUFAs diet (48% corn oil) resulted in a lowered level of SCD1 mRNA expression in the liver when offered to lean or obese Zucker rats as compared to the control group of rats on a normal diet (12% corn oil) (Jones et al., 1996). It has been reported that treatment of 3T3-L1 adipocytes with arachidonic acid suppressed SCD1 expression and enzymatic activity without changing the transcription of the SCD1 gene, although the half-life of SCD1 mRNA was reduced from 25.1 to 8.5 hours. In the same way, the treatment of the cells with linoleic acid (18:2n-6), linolenic acid (18:3n-3) and eicosapentaenoic acid (20:5n-3) also repressed the SCD1 mRNA expression. It led the authors to conclude that PUFAs suppress the expression of SCD1 in mature adipocytes without affecting its transcription but they modify the SCD1 expression by reducing the stability of SCD1 mRNA (Sessler et al., 1996).

The decreased expression of SCD1 by linoleic acid is dose dependent in 3T3-L1 adipocytes (Jones et al., 1996). Moreover, the activity of the SCD promoter, from four different species (mouse, man, pig and sheep) is declined in a dose-dependent manner by supplementing linoleic acid (18:2, n-6). This effect of linoleic acid (18:2, n-6) is dependent on the presence of a PUFA response region in SCD1 gene. In all these species, the PUFA response region of the SCD promoter was indicated with the presence of an active sterol response element (SRE) (Zulkifli et al., 2010). It had already been suggested that SRE in SCD1 promoter mediates the response of SCD1 gene to fatty acids (Ntambi, 1999). PUFAs may control the transcription of SCD since it has been demonstrated that PUFAs show inhibitory effect on SREBP-1c transcription and SREBP-1c mediates the transcription of SCD gene (Yoshikawa et al., 2002). To investigate the effect of PUFAs on liver fats, a cohort of 60 subjects was offered with a diet rich in either PUFAs (linoleic acid). Subjects who utilized a diet rich PUFAs (n-6 PUFAs) were found with remarkably lower fats contents in liver and decreased ratio of 16:1 n-7/16:0 in CE fraction of plasma (Bjermo et al., 2012).

It is now well established that PUFAs control the expression of SCD1 in negative manners and a set of studies has shown consistent data confirming that PUFAs repress SCD1 expression and enzymatic activity. SCD4 isoform is an exception in this regard because its expression is not altered in response to PUFAs (Miyazaki et al., 2003). More efforts are needed to explore the influence of PUFAs on SCD1

expression in human for therapeutic intervention against health threatening metabolic disorders.

10.5.6 Cholesterol:

SCD plays a critical role in maintaining cholesterol homeostasis as its products MUFAs are key elements in CE synthesis in liver and SCD1 ko mice lack CE (Miyazaki et al., 2000). Dietary cholesterol has been reported to induce the expression of SCD1 mRNA but SCD2 expression decreases in the presence of cholesterol (Tabor et al., 1998; Kim et al., 2002b; Nakamura and Nara, 2004). Provision of a diet containing 1% cholesterol resulted in augmentation of hepatic SCD1 expression and oleic acid concentration suggesting that oleic acid is esterified to CE (Landau et al., 1997). This effect of cholesterol on SCD1 expression was independent of other fatty acids added with cholesterol containing diet (Garg et al., 1988). Liver X receptor/retinoid X receptor (LXR/RXR) transcription factors induce various genes such as SCD1 that are involved in the regulation of cellular cholesterol. As a result of HEK 293 cells co-transfection of ATP-binding cassette transporter A1 (ABCA1) and SCD1 or SCD2, ABCA1 mediated cholesterol efflux was inhibited, whilst over expression of SCD1 in chinese hamster ovary (CHO) cells resulted in a 73% decline in cholesterol efflux (Sun et al., 2003).

In a recent study, it has been demonstrated that SCD1 activity enhances cell viability and supplies substrate for acyl-CoA:cholesterol acyltransferase (ACAT)-mediated CE synthesis. In this way it helps to reduce the free cholesterol and minimizes the cholesterol mediated lipotoxicity. Inhibition of SCD1 decreased the biosynthesis of cholesterol esters, suggesting that SCD1 synthesised oleate is important for the synthesis of CE (Paton and Ntambi, 2010). Free Cholesterol concentration increases on inhibition of SCD1 that may result in remodelling the membranes. All these data reveal that SCD1 expression is necessary for synthesis of cholesterol from the esterification of MUFAs. It has been reviewed that cholesterol mediated induction of SCD1 bypasses the mechanism of SREBP-1c maturation (Kim et al., 2002a).

10.5.7 Protein:

Only study has been done to measure SCD1 activity in liver microsomes of monkeys fed on a control or low-protein diet for a period of 12 months. They

demonstrated active hepatic delta 9-desaturase enzyme. The animals fed on low-protein diet were found with an increase in SCD1 activity and had significantly higher oleic acid (18:1n-9) (Marín et al., 2003).

10.5.8 Dietary restriction/lifestyle intervention:

Diet and lifestyle have great impact on the health condition of human beings. Dietary restrictions are being suggested as a remedy for obesity and obesity related disorders. In one study, rats after chronic food restriction showed enhanced SCD1 mRNA expression in various body tissues including liver, epididymal, perirena and subcutaneous adipose tissues. In case of refeeding following a chronic food restriction, the expression of SCD1 was 7-fold higher, while just chronic restriction resulted in 2-fold increase in SCD1 expression. Short fasting showed negative effects and after 72 hours fasting SCD1 expression was found repressed as compared to the control group. The authors hypothesized that elevated expression of SCD1 with dietary restriction and refeeding may be part of a molecular mechanism(s) by which synthesis of fatty acids following caloric restriction augments the susceptibility to obesity and insulin resistance (Turyn et al., 2010).

After weight loss, body tends to increase the metabolic efficiency to recover the fat depots and thermogenesis is halted by a positive feedback control system consisting of signals from depleted adipose tissues exerting suppressive effects on heat generation in the body. SCD1 expression in retroperitoneal white adipose tissues and liver decreased while in epididymal adipose tissues the SCD1 mRNA expression remained unchanged after semi-starvation and refeeding. Similarly, SCD1 expression was enhanced in skeletal muscle after 2 weeks semi-starvation and remained elevated after refeeding. The microsomal SCD1 activity and mRNA expression went parallel with increased MUFAs ratio in muscles. It was supposed that enhanced SCD1 level in skeletal muscle controls DNL and lipid oxidation by desaturating the products of DNL and taking them away from mitochondrial oxidation for increasing lipid storage. Author concluded that SCD1 expression varies in various tissues following starvation (Mainieri et al., 2006). In a study on human, the manipulators measured the expression of SCD1 mRNA in adipose tissues after various diet types, acute weight loss and weight stabilization. SCD1 expression was suppressed following acute weight loss and changed more modestly in response to modified diet composition (Mangravite et al., 2007).

Although sufficient data is not available on the aspect of dietary restriction/lifestyle intervention and SCD1 expression but from the available information, it can be concluded that tissue specific regulation of SCD1 is noted as a result of a particular diet intake in routine life. More work and efforts are needed in this regard to elucidate the mechanism and effect of nutritional status and lifestyle on SCD1 expression.

10.5.9 Alcohol:

The effect of alcohol on SCD1 expression has been investigated by various groups. The results from one group don not corroborate the other group's results. To study the influence of alcohol on fatty acid ratio, rats were fed on ethanol containing food (36% of calories as ethanol) and control group on a diet (containing maltose-dextrin isocalorically substituted for ethanol) for 31 days. The oleate contents in membrane lipids were found elevated while palmitate contents were found decreased in response to chronic administration of alcohol (Cunningham et al., 1982). Another investigation has reported that by increasing alcohol intake, Palmitoleic acid (16:1 n-7) concentration increased in plasma PLs (Mozaffarian et al., 2010). While in another study no difference was observed in the SCD1 mRNA expression in the liver although other lipogenesis genes such as FAS expression was increased remarkably (Wada et al., 2008). Alcohol intake suppresses the expression of SCD1 has also been reported. The results came with interpretation that SCD1 activity reduced notably in the microsomes of rats that were fed ethanol. There was a 80% decline in the desaturation of palmitoyl-CoA while stearoyl-CoA desaturation activity was totally lost (Umeki et al., 1984). The influence of chronic alcohol utilization on the expression of SCD1 mRNA is reported to vary in various studies because both SCD1 and SCD2 expression level was observed not significantly increased in response to chronic administration of alcohol (Tomita et al., 2004).

The effect of alcohol consumption on SCD1 mRNA expression is not well studied so no conclusive data is available. Further investigation is required to explore the influence of alcohol on SCD1 expression in human.

10.6 Other factors that may influence SCD:

10.6.1 Cold:

Poikilothermic animals, with varying internal temperature considerably as a consequence of variation in the external environmental temperature, respond to decreasing temperature by increasing desaturation of their phosphoglyceride to restore the fluidity of rigidified membranes by cold (Tiku et al., 1996; Cossins et al., 2002).

The SFAs desaturation to increase the abundance of cis-MUFAs has a remarkable influence on membrane fluidity as cis-unsaturated fatty acids decrease the temperature for membrane lipid phase changes (Hodson and Fielding, 2013). A handsome set of investigations has been done on carp to figure out the mediation of SCD1 expression in response to cold. When carp were transferred from a higher temperature 30°C to a lower temperature 10°C, an 8 to 10-fold increase in the specific activity of the microsomal SCD1 was observed. After 48-60 hours, a 10-fold increase in the cold-induced expression of SCD1 gene transcription was noted (Tiku et al., 1996; Trueman et al., 2000) and hepatic SCD1 and SCD2 induction was noted in response to cold (Polley et al., 2003). The same trend of increased expression of SCD1 to respond external low temperature has reported in milkfish and grass carp (Hsieh and Kuo, 2005).

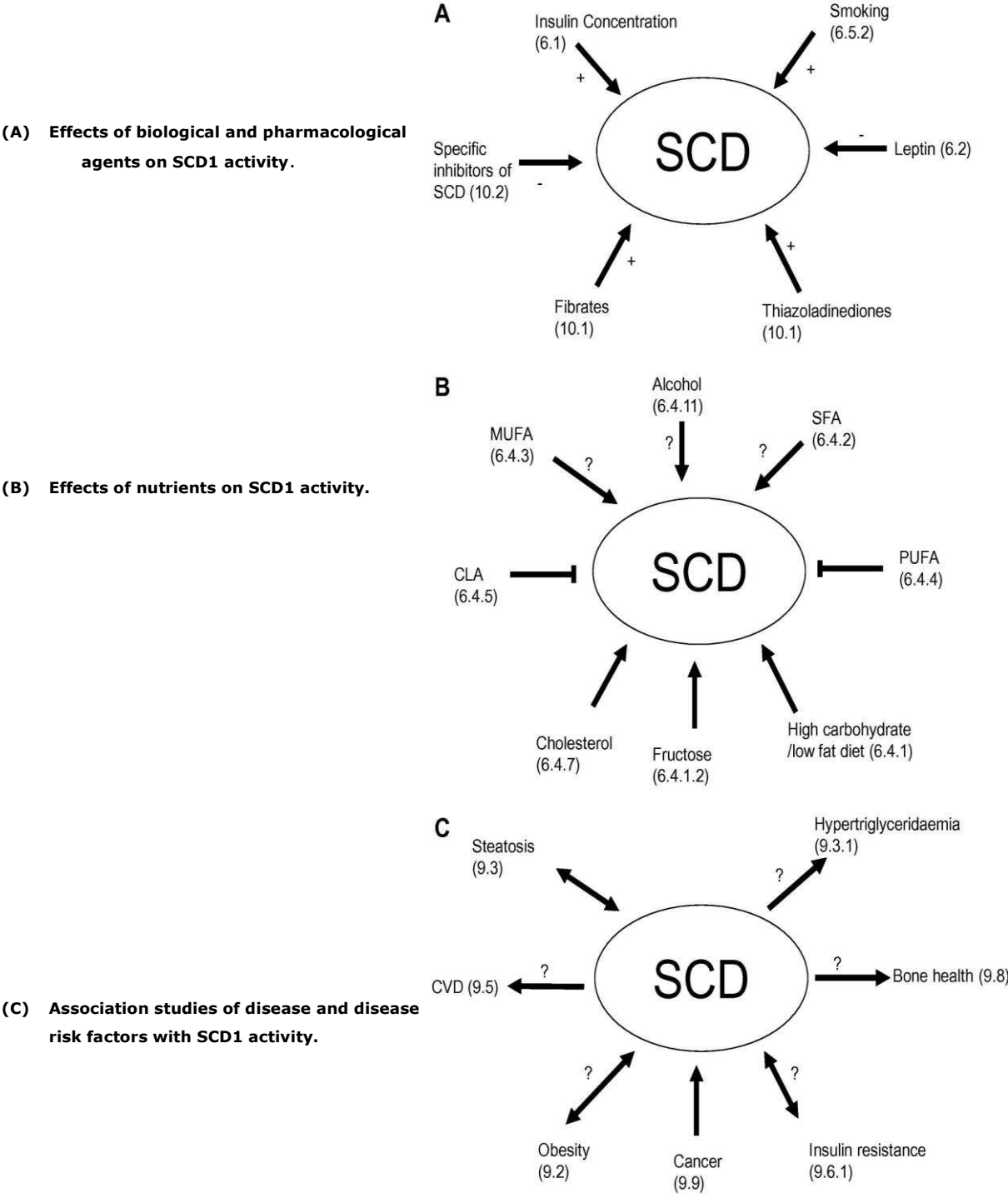
Whether human respond to external temperature changes in the same way as has been documented in fish and other animals is still unknown and demands efforts to investigate all these aspects.

10.6.2 Shear stress:

Vascular endothelium is constantly exposed to a variety of forces and plays a key role in detecting the changes in chemical and physical properties in blood flow to maintain physiological homeostasis in blood vessels. Shear stress resulted from the friction of blood flow affects the structure and function of endothelial cells leading to atherosclerotic lesions in the vessels. Recent modern techniques for example genomic and proteomic approaches have shown that shear stress may mediate distinct changes in genes expressed in ECs. As a result of shear stress, stimulation of cell metabolism and increased membrane fluidity of vascular endothelial cells has been reported. Since SCD1 products are important to maintain membrane fluidity and functions, so the expression of SCD1 was

measured in response to shear stress on ECs. Laminar flow effects were measured on SCD1 expression by using *in vitro* and *in vivo* models. Laminar shear stress markedly elevated the level of SCD1 expression that was found to be mediated by PPARc pathway. Author suggested that these alterations in SCD1 expression are a mechanism that helps to neutralize the effects of shear stress on ECs (Haidekker et al., 2000; Butler et al., 2001; Garcia-Cardena et al., 2001; Qin et al., 2007).

Fig. 7: Factors affecting SCD1 activity:



Arrows represent the putative direction of effect and a blunted line shows an inhibitory effect. The + or - symbols show up or down-regulation of SCD1. Double ended arrow indicates uncertainty regarding cause or effect. Question mark shows that further evidence is required to be certain of an association. Abbreviations: SCD, stearyl-CoA desaturase; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; CLA, conjugated linoleic acid; CVD, cardiovascular disease (Hodson and Fielding, 2013).

OBJECTIVE

Taking into account all these pieces of information and available data of ALS patients and animal model of ALS, the prevalence of metabolic dysfunctions in ALS is now out of the question. These metabolic dysfunctions are the result of alterations in the expression of a number of genes that modulate the metabolic pathways. Lipids being highly energy rich have been investigated extensively in regards to combat enhanced energy requirement in ALS patients. Food rich in lipids and high ketogenic diets ameliorate the pathological conditions and prolong life expectancy to a certain extent. Moreover, lipids comprise a large number of species with varying functions related to physiology, maintenance and energy provision in any organism. The metabolic alternations observed in ALS patients and animal models can coincide with the modified levels of various lipid species particularly in affected tissues. Since muscles are the tissues firstly affected during this process, it led us to focus on the role of metabolic dysfunctions in muscles as leading preliminary change in this pathology.

SCD1 is a key enzyme that catalyses the synthesis of MUFAs from SFAs. Gene profiling studies have shown that SCD1 expression is modified in ALS mouse models and human. It is also known that SCD1 expression in muscles is altered in response to the modifications in lipids abundance. The mice with disrupted SCD1 show an enhanced level of the genes involved in the β -oxidation of fatty acids and repressed level of the genes involved in lipogenesis. They are lean, have reduced body adiposity and show augmented energy expenditure the traits observed in ALS. In spite of common features at the metabolic level in SCD1 knockout mice but it is yet not known whether a decrease in SCD1 expression could be implicated by any means in the maintenance of muscle function and, perhaps, in motor neuron degeneration.

In this study we investigated the implication of SCD1 in skeletal muscle functions at normal condition and their capacity to recover following a nerve injury. We also analysed the changes in fatty acids related to SCD1 activity in the mouse model of ALS. The findings of this study will provide the insight into the comprehension of metabolic alterations contributing to ALS.

In the first chapter we established a relationship between neurodegeneration and metabolic dysfunctions. So, first we characterized the expression of SCD1 in muscles from mutant SOD1 mice as well as in experimentally denervated

muscles. Second, we studied the muscle phenotype of mice deficient in SCD1. Third, we analyzed the impact of the absence of SCD1, as obtained by both genetic and pharmacological approaches, on the restoration of muscle function in response to the transient nerve lesion (see PLoS ONE article).

In the second chapter, we analysed the lipid profile of mutant SOD1 mice a model of ALS. Since lipids are diverse in size and functions, their tissue specific prevalence, alteration and type can explore the new horizon towards deciphering the pathological mechanism of ALS. We focused the periphery; serum and liver for analysing the composition of FAs in neutral lipids *e.g.*, mainly TAG and cholesterol esters (see manuscript in preparation). Moreover, in this part of the study, we addressed from a more general point of view the implication of fatty acids in nervous system disorders, in the form of a review article.

RESULTS

Chapter 1:

**Implication of SCD1 in muscle function recovery following a
nerve injury**

1. SCD1 knockout mouse or SCD inhibition:

Two approaches can be applied to washout or inhibit the SCD expression and activity in the mouse; genetic deletion or pharmacological inhibition. There are some murine models that lack SCD1 expression globally or tissues specific elimination of SCD1 expression. In our study we used globally knockout SCD1 gene in C57BL/6 mouse strain and mice with SCD inhibition pharmacologically by a synthetic molecule. Both mouse models share common features of altered metabolic state and apparent phenotypic characters. SCD1 knockout mouse general features are described in detail in the following portion. These mice have been widely used to investigate the various metabolic processes and related disorders (Ntambi et al., 2002; Rahman et al., 2003, 2005; Miyazaki et al., 2004b, 2009; Dobrzyn et al., 2005a, 2005b, 2005c; Flowers et al., 2007; Hyun et al., 2010) .

1.1. SCD1 knockout mouse:

The SCD1 gene targeted mouse model with the disrupted SCD1 gene has been documented that manifests lost SCD1 expression and activity globally. The mice lacking SCD1 expression and activity are viable with normal growth on chow diet; they are lean, represent reduced body weight and resistance to obesity. They exhibit metabolic abnormalities, skin problems (alopecia) and narrow eye fissure with malfunctioning sebaceous and meibomian glands. They suffer from the deficiency of TGs, CEs and wax esters in skin and eyelid. Free cholesterol level in the eyelid and the skin is higher than wild type mice (Miyazaki et al., 2001b; Ntambi et al., 2002). They exhibit resistance to obesity caused by diet or leptin deficiency (Dobrzyn and Ntambi, 2004; Miyazaki et al., 2005). The *Scd1*^{-/-} mice have high energy expenditure and oxygen consumption that hinder the weight gain. Due to malfunctioning of sebaceous and meibomian glands, skin barrier does not function properly to regulate thermohomeostasis in the body and may somehow result in alopecia (Cannon and Nedergaard, 2011; Sampath and Ntambi, 2011). Thermogenesis in *Scd1*^{-/-} mice is upregulated due to over expression of genes involved in lipolysis but on exposure to low temperature these mice suffer from hypothermia and hypoglycemia with lower core temperature. Extragenously provided triolein but not tristearin helped to recover thermogenesis and longer core temperature maintenance (Lee et al., 2004).

The *Scd1*^{-/-} mice show low level of ceramide (40 % low), sphingomyelin, fatty acyl-CoA, serine palmitoyltransferase (SPT) and free fatty acids in soleus and red gastrocnemius muscle. In contrast, these *Scd1*^{-/-} mice have elevated level of Carnitine palmitoyltransferase 1 (CPT1) mRNA and activity in parallel to an enhanced rate of β -oxidation in these muscles (Dobrzyn et al., 2005a). In summary, SCD1 deficiency suppresses ceramide synthesis by increasing β -oxidation and reducing serine palmitoyltransferase (SPT) expression in oxidative muscles. Increased insulin sensitivity and augmented plasma ketones level are caused by SCD1 depletion in mice while decreased plasma insulin and leptin are also observed in these mice (Ntambi et al., 2002). The muscles of SCD1 deficient mice have lower level of insulin while increased glucose uptake. Over expression of insulin receptors (IR) and its substrate 1, 2 (IRS1-2) are main feature of these muscles and brown adipose tissues (Rahman et al., 2003). Deficiency of SCD1 results in activation of AMP-activated protein kinase (AMPK) in liver and skeletal muscles. The activation of AMPK negatively regulates the synthesis of fatty acids and cholesterol. AMPK activation evokes catabolic pathways such as β -oxidation of FAs to generate ATP (Dobrzyn et al., 2004, 2005a).

1.2. SCD1 inhibition:

SCD1 inhibition by various molecular strategies has been documented in a number of studies. There are molecules that inhibit SCD1 globally and tissue specific inhibition can also be achieved by particular products available in the market. Basically these products have been developed and evaluated as drugs against obesity and metabolic syndromes (Issandou et al., 2009; Koltun et al., 2009b; Li et al., 2009a; Léger et al., 2010; Powell et al., 2010; Oballa et al., 2011). The specific inhibitors of SCD1 available are not devoid of side effects of this SCD1 inhibition. The global inhibition of SCD1 yields skin and eye related problems and this led to the development of tissue specific SCD1 inhibitors particularly hepatic SCD1 targeting inhibitors (Ramtohl et al., 2010; Oballa et al., 2011).

1.3. Inhibitors:

The very first study concerning SCD1 inhibition by antisense oligonucleotides (ASOs) came out in 2005, which evaluated an ASO to inhibit SCD1 expression and activity *in vivo*. These antisense oligonucleotide inhibitors were SCD1 specific and they successfully reduced the expression of SCD1,

suppressed fatty acid synthesis and enhanced fatty acid oxidation in primary murine hepatocytes. The reduced 16:1 n-7/16:0 and 18:1 n-9/18:0 ratio, and lower TGs abundance in tissues and plasma were noted after intervention (Jiang et al., 2005). This ASOs inhibitor effectively inhibited the target gene in rat also with the same phenotype (Gutiérrez-Juárez et al., 2006). The pharmacological inhibition of SCD1 is adopted to deal with obesity and metabolic syndromes but this approach is not devoid of other side effects, for example, skin problems, atherosclerosis and hyperthermogenesis. Sometimes this inhibition can result in SFAs accumulation in plasma, tissues, and repressed plasma TGs concentration (Brown et al., 2008). A persistent effort has been done to develop SCD1 inhibitors to inhibit SCD1 expression and activity systematically as observed in genetic ablation of *Scd1* gene (Liu et al., 2007; Atkinson et al., 2011; Isabel et al., 2011; Lachance et al., 2012a; Ortinou et al., 2012). To avoid the side effects related to global inhibition of SCD1, tissues specific SCD1 inhibitors have emerged out. Most of these inhibitors have been designed in such way that they target SCD1 expression in particular tissue of interest such as liver, adipose tissue (Koltun et al., 2009a; Lachance et al., 2012b; Kurikawa et al., 2013).

2. SCD1 expression in muscles:

Skeletal muscles use fat as fuel and are key tissues for fatty acid oxidation (Van Hall et al., 2002) and glucose disposal mediated by insulin (Baron et al., 1988). SCD1 expression is elevated in the muscles of obese subjects. Muscles in case of obesity provide a locality for fat deposition with suppressed fatty acid oxidation and elevated triacylglycerol esterification resulting from enhanced lipogenic gene SCD1 expression (Hulver et al., 2005).

The partition of fatty acids by SCD1 in the skeletal muscles plays an important role in diminishing fatty acids induced insulin resistance. It has been documented that one hour of acute exercise prior to lipid intake is sufficient to halt insulin resistance. The elevated expression of SCD1 in response to exercise increased the TAG level and decreased ceramide and DAG quantity. The study suggested that exercise facilitated the fatty acids partition to enhance TAG synthesis by SCD1 increased expression (Schenk and Horowitz, 2007). Similarly, chronic exercise increases SCD1 expression to facilitate the muscle adaptation towards increased oxidation level on long term exercise. Only the soleus muscle of train rats displayed a high level of SCD1 mRNA and protein quantity along with

elevated 18:1n-9/18:0 ratio. This oxidative muscle also showed increased expression of genes involved in fatty acid oxidation. On the basis of these data, it can be concluded that during training SCD1 increased expression is mandatory for TAG synthesis and muscle adaptation for a long-term exercise (Dobrzyn et al., 2010b). It has also been found in human that SCD1 expression increases in athletes with increased level of intramyocellular triacylglycerol (IMTAG) (Bergman et al., 2010).

Some other studies have emerged with contradictory suggestions that muscular over expression of SCD1 causes aberrant partitioning of fatty acids and increases TAG synthesis leading to metabolic syndrome. The authors found that skeletal muscles of obese objects had 3-fold higher SCD1 expression level as compared to the non-obese lean objects skeletal muscles. This expression was observed positively correlated with body mass index (BMI), IMTAG synthesis but oxidation of fatty acids was noted negatively correlated with this SCD1 increased expression. They tried to confirm these data by *in vitro* approach by using myocytes from obese and lean donors. They noted that primary myocytes from obese subjects had high SCD1 mRNA level and protein leading to increased incorporation of fatty acids into TAG. But remarkably suppressed fatty acids oxidation rate was observed when compared to the data from primary skeletal myocytes from lean donors. Interestingly, the myocytes from lean objects on incubation with oleate or overexpression of SCD1 yielded the same outputs: elevated incorporation of fatty acids into TAG and decreased fatty acid oxidation. The authors concluded that higher level of SCD1 in skeletal muscles led to metabolic disorders by increasing IMTAG level and decreasing oxidation of fatty acids (Hulver et al., 2005).

Systemic Down-Regulation of Delta-9 Desaturase Promotes Muscle Oxidative Metabolism and Accelerates Muscle Function Recovery following Nerve Injury

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Abstract

The progressive deterioration of the neuromuscular axis is typically observed in degenerative conditions of the lower motor neurons, such as amyotrophic lateral sclerosis (ALS). Neurodegeneration in this disease is associated with systemic metabolic perturbations, including hypermetabolism and dyslipidemia. Our previous gene profiling studies on ALS muscle revealed down-regulation of delta-9 desaturase, or SCD1, which is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids. Interestingly, knocking out SCD1 gene is known to induce hypermetabolism and stimulate fatty acid beta-oxidation. Here we investigated whether SCD1 deficiency can affect muscle function and its restoration in response to injury. The genetic ablation of SCD1 was not detrimental *per se* to muscle function. On the contrary, muscles in SCD1 knockout mice shifted toward a more oxidative metabolism, and enhanced the expression of synaptic genes. Repressing SCD1 expression or reducing SCD-dependent enzymatic activity accelerated the recovery of muscle function after inducing sciatic nerve crush. Overall, these findings provide evidence for a new role of SCD1 in modulating the restorative potential of skeletal muscles.

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Introduction

In mammals, the contraction of voluntary skeletal muscles is under the control of motor neurons whose cell bodies are located in the spinal cord and the brainstem. These so-called lower motor neurons directly communicate with muscle fibers through the neurotransmitter acetylcholine at the neuromuscular junctions. The progressive functional deterioration of this neuromuscular axis is typically found in neurodegenerative conditions such as amyotrophic lateral sclerosis (ALS). This disease, which is the most frequent adult-onset form of motor neuron disease, is characterised by motor neuron death, skeletal muscle atrophy and paralysis [1]. Studies conducted on genetic animal models of ALS showed that the process leading to motor neuron degeneration is

not cell-autonomous but involves defects in other cell types than neurons [2–5]. Consistent with this notion, ALS neurodegeneration is also associated with systemic defects, including hypermetabolism and dyslipidemia, which are observed in both patients and animal models [6–8]. Particularly, transgenic mice overexpressing a mutated form of Cu/Zn superoxide dismutase (SOD1), linked to familial ALS, are in energy deficit and have decreased adipose tissue stores. These deficiencies appear to be elicited by increased energy expenditure (that is to say, hypermetabolism), and due to an increased consumption of nutrients by skeletal muscles [9,10].

To gain insight into the relationships between neurodegeneration and metabolic dysfunction, we recently analyzed the gene expression profiles of skeletal muscles from mutant SOD1 mice

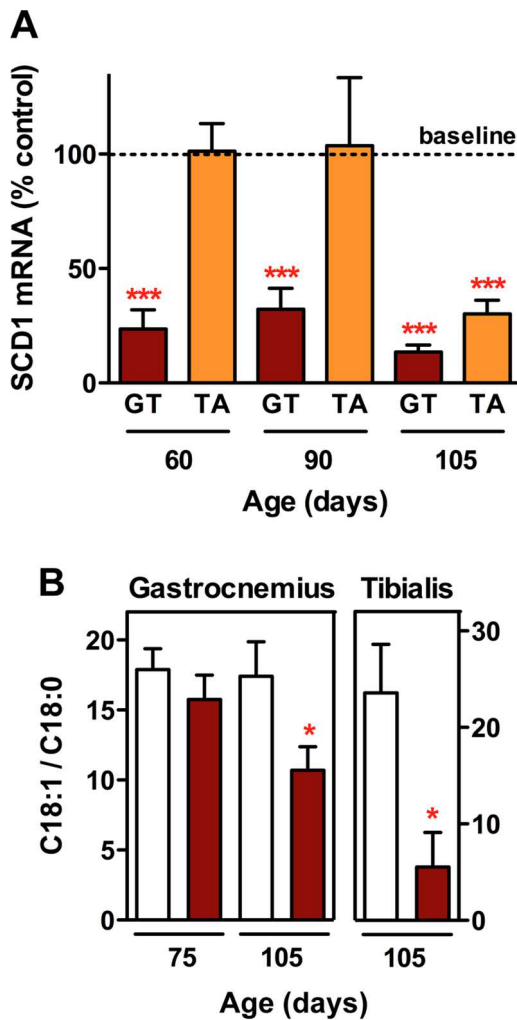


Figure 1. SCD1 expression and activity in ALS mouse muscle. (A) Time course of SCD1 expression in gastrocnemius (GT, brown columns) and tibialis anterior (TA, orange columns) from SOD1(G86R) mice at indicated ages. Wild-type expression is represented by 100% baseline. *** $P < 0.001$ (One sample t -test, $n = 5-11$). (B) C18:1/C18:0 fatty acid ratio in gastrocnemius and tibialis anterior from SOD1(G86R) mice (brown columns) and wild-type littermates (white columns) at indicated ages. * $P < 0.05$ (1-way ANOVA followed by Bonferroni's multiple comparison test for gastrocnemius, and unpaired t -test for tibialis anterior, $n = 3-10$). doi:10.1371/journal.pone.0064525.g001

and patients with sporadic ALS [11,12]. We found a decrease in the expression of stearoyl-CoA desaturase-1 (SCD1), an enzyme that introduces the first *cis* double bond in the delta-9 position of saturated fatty acyl-CoA substrates. The preferred substrates are palmitoyl-CoA (C16:0) and stearoyl-CoA (C18:0), which are converted to palmitoleoyl-CoA (C16:1) and oleoyl-CoA (C18:1), respectively [13]. These monounsaturated fatty acids are the major constituents of complex lipids such as diacylglycerols, phospholipids, triglycerides, wax esters and cholesterol esters. Interestingly, the targeted disruption of the mouse SCD1 gene triggers an increase in the expression of genes involved in the β -oxidation of fatty acids and a decrease in the expression of genes involved in lipogenesis [14]. Therefore, these SCD1 knockout mice exhibit augmented energy expenditure and reduced body adiposity [14], a situation that is reminiscent of the metabolic phenotype of mutant SOD1 mice [9]. Despite sharing common

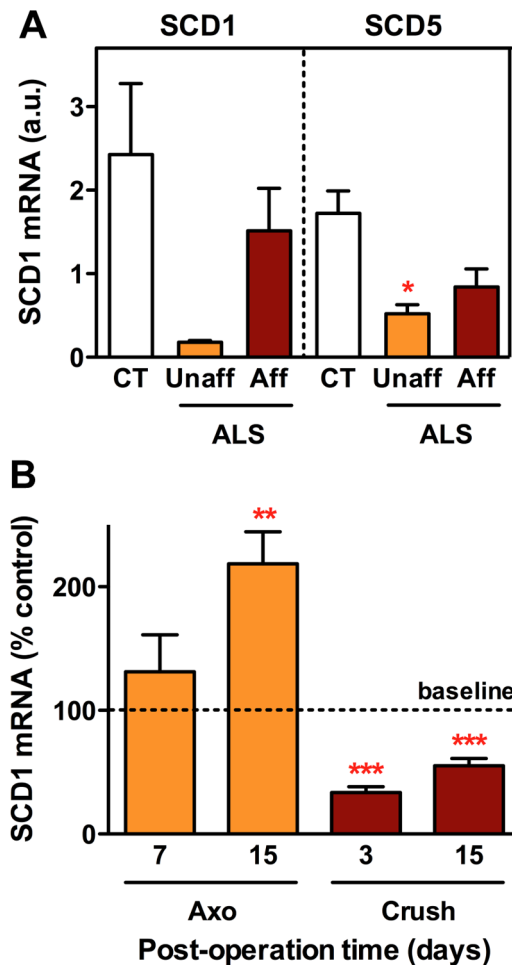


Figure 2. SCD1 expression in ALS patient muscle and after nerve injury. (A) Expression of SCD1 and SCD5 in deltoid muscle biopsies from ALS patients and healthy subjects (CT, white columns), as identified by microarray analysis of the database deposited at [http://www.ebi.ac.uk/arrayexpress/\(accession number E-MEXP-3260\)](http://www.ebi.ac.uk/arrayexpress/(accession number E-MEXP-3260) [12]. ALS samples were obtained from muscle not clinically or electromyography affected (Unaff, orange columns) and from muscle with advanced pathology, characterized by reduced strength and neurogenic electromyography pattern (Aff, brown columns). * $P < 0.05$ (1-way ANOVA followed by Tukey's multiple comparison test, $n = 4-10$). (B) Expression of SCD1 in gastrocnemius following sciatic nerve axotomy (Axo) or crush at indicated post-operation days. Contralateral muscle expression is represented by 100% baseline. ** $P < 0.01$, *** $P < 0.001$ (One sample t -test, $n = 4-10$). doi:10.1371/journal.pone.0064525.g002

features at the metabolic level, it is not known whether a decrease in SCD1 expression could be implicated by any means in the maintenance of muscle function and, perhaps, in motor neuron degeneration. In this work, first we characterized the expression of SCD1 in muscles from mutant SOD1 mice as well as in experimentally denervated muscles. Second, we studied the muscle phenotype of mice deficient in SCD1. Third, we analyzed the impact of the absence of SCD1, as obtained by both genetic and pharmacological means, on the recovery of muscle function in response to transient nerve lesion. We conclude that the systemic down-regulation of SCD1 promotes muscle oxidative metabolism and accelerates muscle function recovery after nerve injury, thus providing evidence for a new role of this enzyme in modulating the restorative potential of skeletal muscles. These findings therefore

may be relevant to pathological conditions affecting the lower motor neurons.

Materials and Methods

Animals

FVB/N males overexpressing the murine G86R SOD1 mutation [15], and C57BL/6 males knockout for the SCD1 gene (The Jackson Laboratory, Bar Harbor, ME) were maintained in our animal facility at 23°C with a 12 hours light/dark cycle. They had water and regular A04 rodent chow *ad libitum*. SOD1(G86R) mice were 60-, 75-, 90- and 105 days of age. SCD1 knockout mice were 4–6 months old. The corresponding non-transgenic male littermates served as controls. To induce peripheral nerve injury, mice were anesthetized with 100 mg/kg body mass ketamine chlorhydrate and 5 mg/kg body mass xylazine. The sciatic nerve was exposed at the midhigh level, and crushed with a fine forceps for 30 s or sectioned 3 mm long with microscissors. Skin incision was sutured, and mice were allowed to recover. Hind limbs contralateral to the lesion served as controls [16]. To induce SCD deficiency pharmacologically, 4–6 months old mice were fed with A04 chow *ad libitum* containing 3-(5-methyl-[1,3,4]thiadiazol-2-yl)-6-[4-(2-trifluoromethyl-phenoxy)-piperidin-1-yl]-pyridazine, or MF-438 (Prestwick Chemical, Illkirch, France), which is an orally bioavailable inhibitor of SCD enzymatic activity [17]. The drug regimen was prepared to contain 0.00625% (w/w) MF-438 (Safe, Augy, France), which provided a daily dose of 10 mg/kg body mass, as calculated on a basis of 4 g of food intake per day and 25 g of averaged body mass.

Ethics statement

Experiments followed current European Union regulations (Directive 2010/63/EU), and were performed by authorized investigators (license from *Prefecture du Bas-Rhin* No. A67-402 to AH, and No. A67-118 to FR), after approval by the ethics committee of the University of Strasbourg (license from CRE-MEAS No. AL/01/20/09/12, and No. AL/15/44/12/12).

Quantitative RT-PCR

Total RNA was prepared following standard protocols. Briefly, each frozen sample was placed into a tube containing a 5-mm stainless steel bead. Working on ice, 1 mL Trizol reagent (Invitrogen, Groningen, The Netherlands) was added, and homogenisation was performed in a TissueLyser (Qiagen, Valencia, CA) at 30 Hz for 3 min twice. RNA was extracted with chloroform/isopropyl alcohol/ethanol and stored at -80°C until use. One μg of total RNA was used to synthesize cDNA using Iscrip reverse transcriptase (BioRad Laboratories, Marnes La Coquette, France) and oligo-dT primer as specified by the manufacturer. Gene expression was measured using the SYBR green reagent ($2\times$ SYBR Green Supermix; Bio-Rad Laboratories) following the manufacturer's instructions on a Bio-Rad iCycler. PCR was performed in optimized conditions: 95°C denatured for 3 min, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C . Primers (Eurogentec, Seraing, Belgium) were as follows: acetylcholine receptor α -subunit (AChR- α), forward 5'-ccacagactcagg-gagaag-3', reverse 5'-aacggtgggtgtgttgatg-3'; acetylcholine receptor γ -subunit (AChR- γ), forward 5'-gagagccactcgaagacac-3', reverse 5'-gaccaacctcatcctccta-3'; acetylcholine receptor ϵ -subunit (AChR- ϵ), forward 5'-caatccaatccagacactg-3', reverse 5'-ccctgcttctcctgacactc-3'; muscle-specific receptor tyrosine kinase (MuSK), forward 5'-ttcagcgggactgagaaact-3', reverse 5'-tgtcttcacgctcagaatg-3'; peroxisome proliferator-activated receptor α (PPAR α), forward 5'-cacgcatgtgaaggctgtaa-3', reverse 5'-

gtccgatcacactgtg-3'; peroxisome proliferator-activated receptor γ , coactivator 1- α (PGC1- α), forward 5'-tgctgctgtcctgtttt-3', reverse 5'-ccctgccattgtaagacc-3'; pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4), forward 5'-cgcttagtgacactccttcg-3', reverse 5'-cttctgggctcttctcatgg-3'; ribosomal RNA 18S (18S), forward 5'-cgtctgccatcaactcttcg-3', reverse 5'-ttcttggatgtgtagccg-3'; SCD1, forward 5'-cctacgacaagaacattcaatcc-3', reverse 5'-cgtctcaagtctct-taactct-3'. Relative quantification was achieved by calculating the ratio between the cycle number (Ct) at which the signal crossed a threshold set within the logarithmic phase of the gene of interest and that of the 18S reference gene. Ct values were the means of duplicates.

Gas chromatography

Muscle samples were weighed, and disrupted with TissueLyser twice at 30 Hz for 3 min each using stainless steel beads and racks precooled at -80°C . After removing the beads, 1 mL ice-cold Ripa buffer was added per 100 mg of disrupted tissue, and samples were centrifuged at 2000 g for 10 min at 4°C . Two mg of proteins, according to the bicinchoninic acid assay in the supernatants, was mixed with 2.5 mL chloroform/methanol (1:1). After vortex and sonication, samples were incubated for at least 2–3 hours at 4°C , and centrifuged at 2000 g for 10 min at 4°C . Supernatants were collected into glass tubes with a Pasteur pipette, and dried under nitrogen. Afterwards, the non-polar lipid fraction was obtained by separation on Sephadex columns. In the case of blood samples, they were collected on heparinized tubes by heart puncture from anesthetized mice, placed on ice, and centrifuged at 4000 rpm for 4 min at 4°C . Total lipids were extracted from plasma following a modified version of the Bligh and Dyer method [18]. Briefly, methanol/chloroform solution was added to plasma. Centrifugations were performed after addition of chloroform and KCl. Lower phases were collected and mixed with methanol. After a second centrifugation, the lower phase containing the lipids was collected on glass tubes. Extracted lipids from muscle and plasma were transmethylated by adding a mix of methanol and KOH. After collecting the samples in heptane, the so-generated fatty acid methyl esters were submitted to gas chromatography by using a Varian 3400 CX chromatograph fitted with a WCOT fused silica capillary column of 100 m \times 0.25 mm \times 0.20 μm (coated with polar highly substituted cyanopropyl CP-SIL 88 phase). The injection volume was 1 μL , and the split ratio was set at 1:1. The temperature gradient in the oven ranged from 80 to 220°C at a rate of $4^{\circ}\text{C}/\text{min}$, and helium was the gas carrier. The temperature of the flame ionization detector was set at 270°C . Peaks were identified by retention time and compared to a standard mix of fatty acid methyl esters (Supelco 37 and Supelco PUFA-2 Animal Source; Sigma-Aldrich, Saint-Quentin Fallavier, France). Data were expressed as relative percentages.

Muscle grip strength

Muscle strength was determined using the grip test (Bioseb, Chaville, France). Animals were placed over a metallic grid that they instinctively grab to try to stop the involuntary backward movement carried out by the manipulator until the pulling force overcomes their grip strength. After the animal loses its grip, the strength-meter scores the peak pull force. Strength was measured independently in hind limbs ipsi- and contralateral to the nerve lesion, and the mean of 3 assays was scored for each animal. Peak force was normalized to body mass.

Muscle histochemistry

The standard histochemical assay for succinate dehydrogenase (SDH) was used to distinguish between oxidative and non-

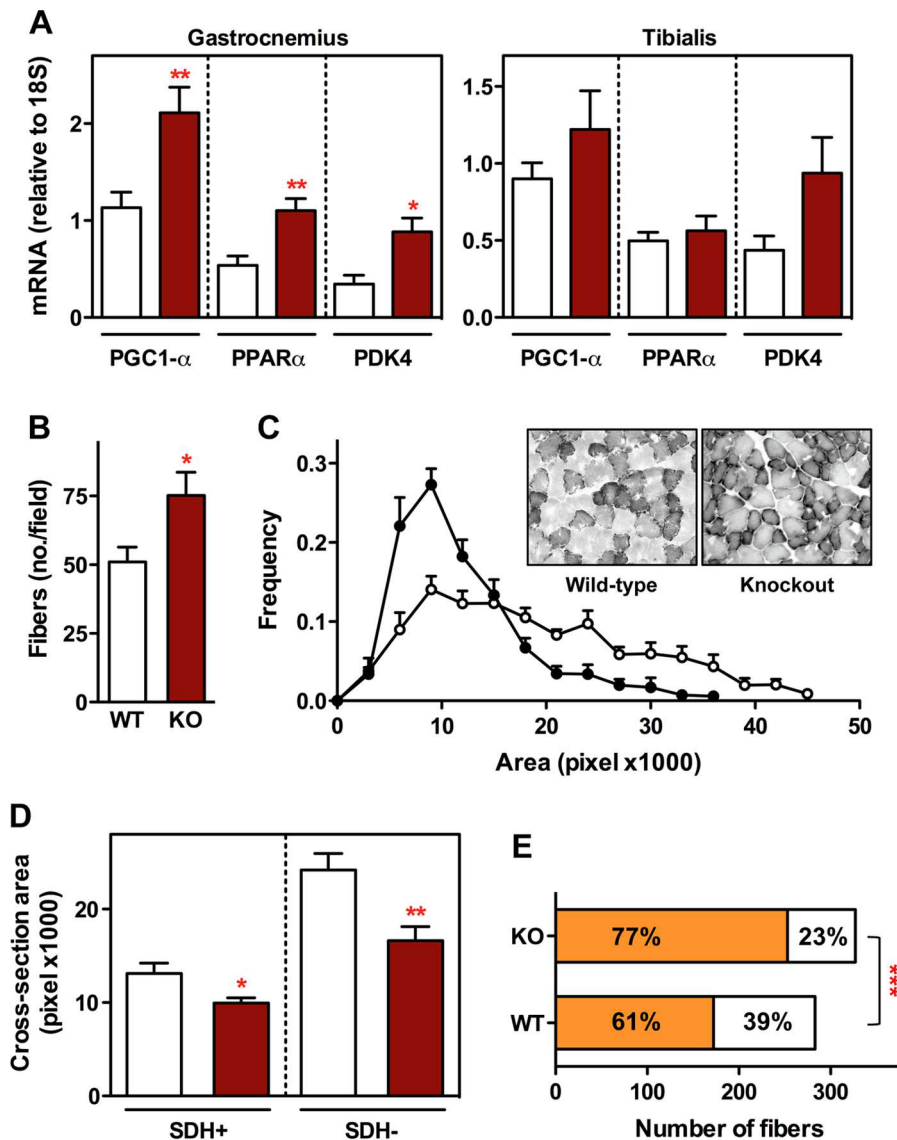


Figure 3. Metabolic phenotype of muscle from SCD1 knockout mice. (A) Expression of PGC1- α , PPAR α and PDK4 in gastrocnemius and tibialis anterior from SCD1 knockout mice (brown columns) and wild-type littermates (white columns). * $P < 0.05$, ** $P < 0.01$ (Unpaired t-test, $n = 3-11$). (B) Number of muscle fibers in tibialis anterior from SCD1 knockout mice (KO, brown column) and wild-type littermates (WT, white column). * $P < 0.05$ (Unpaired t-test, $n = 7-10$). (C) Distribution of the calibers of muscle fibers in tibialis anterior from SCD1 knockout mice (327 fibers, black circles) and wild-type littermates (283 fibers, white circles). Representative microphotographs of wild-type and knockout tibialis anterior are shown. (D) Averaged cross-sectional area of SDH-positive and SDH-negative fibers in tibialis anterior from SCD1 knockout mice (brown columns) and wild-type littermates (white columns). * $P < 0.05$, ** $P < 0.01$ (Unpaired t-test, $n = 7-10$). (E) Number of SDH-positive (orange bars) and SDH-negative fibers (white bars) in tibialis anterior from SCD1 knockout mice (KO) and wild-type littermates (WT). *** $P < 0.001$ (Chi-square test, $n = 283-327$). doi:10.1371/journal.pone.0064525.g003

oxidative muscle fibers. Fourteen- μ m serial sections were obtained by cutting isopentane fresh-frozen tibialis anterior muscles perpendicular to the muscle axis on a cryostat at -20°C (Leica, Nanterre, France). Sections were fixed with acetone for 15 min, air-dried and incubated for 30 min at 37°C in a solution containing 10 g/L sodium succinate, 2 g/L nitro blue tetrazolium and 0.025 g/L phenazine methosulfate. After washing, sections were mounted with aqueous mounting medium (Dako, Trappes, France). Microphotographs were obtained at a magnification of $\times 200$ (Nikon, Champigny sur Marne, France) on five non-adjacent sections per animal. Counts were performed using National Institutes of Health IMAGE version 1.62 software.

Indirect calorimetry

O_2 consumption and CO_2 production were measured using an open-circuit indirect calorimetry system (Klogor, Lannion, France), as previously described [9]. Concentrations of O_2 and CO_2 in the outgoing air were successively measured in five different cages. The system was rinsed for 90 s between each measurement. Final values of gas concentrations were the mean of 10 measures obtained during 40 s. Each cage was sampled every 11 min, and one cage was left vacant as reference of ambient gas concentrations. Measurements were performed continuously over 23 hours and a half, a 30-min period being required for calibration of the O_2 and CO_2 analyzers. In total, 127 measures

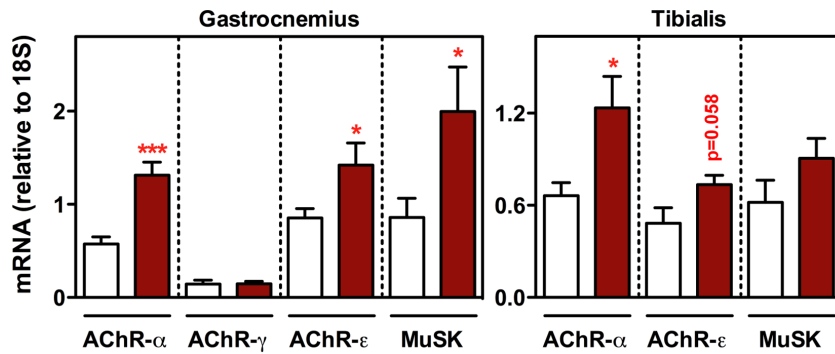


Figure 4. Gene expression specific to the motor end plate in SCD1 knockout mice. Expression of AChR- α , AChR- γ , AChR- ϵ and MuSK in gastrocnemius and tibialis anterior from SCD1 knockout mice (brown columns) and wild-type littermates (white columns). * P <0.05, *** P <0.001 (Unpaired t-test, n = 4–11).

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were collected per day and mouse. The respiratory quotient was the ratio of CO₂ production over O₂ consumption.

Electromyography

Recordings were obtained with a standard electromyography apparatus (Dantec, Les Ulis, France), in accordance with the guidelines of the American Association of Electrodiagnostic Medicine. Mice were anesthetized as indicated above and kept under a heating lamp to maintain a physiological muscle temperature (at about 31°C). A concentric needle electrode (no. 9013S0011, diameter 0.3 mm; Medtronic, Minneapolis, MN) was inserted in the gastrocnemius, and a monopolar needle electrode (no. 9013R0312, diameter 0.3 mm; Medtronic) was inserted into the tail of the mouse to ground the system. Each muscle was monitored in four different regions, and the degree of denervation was scored as the number of regions with spontaneous activity expressed in percentage. Spontaneous activity was differentiated from voluntary activity by visual and auditory inspection. Only spontaneous activity with a peak-to-peak amplitude of at least 50 μ V was considered to be significant.

Statistical analysis

Unless otherwise indicated, data are expressed as the mean \pm SEM. PRISM version 5.0a software (GraphPad, San Diego, CA) was used for statistical analysis. Tests are indicated in the legends under the figures. Differences with P -values of at least less than 0.05 were considered significant.

Results

SCD1 expression is altered in ALS muscle

On the basis of our previous microarray data, obtained from a transgenic mouse model of mutant SOD1-linked familial ALS [11], in this study we investigated the significance of the down-regulation of SCD1 for the metabolic capacity of muscles and their response to injury. The expression of SCD1 in the gastrocnemius of SOD1(G86R) mice, which are affected by a progressive denervation atrophy [19], was already diminished at 60 days of age. In this respect, it is noteworthy to mention that our previous electromyography studies on this mouse line revealed that the amplitudes of the compound muscle action potentials, a reduction of which typically reflects a decrease in the number of functional motor units, were normal at the age of 75 days. In addition, mice did not present at this age any abnormal spontaneous electrical activity, which would have reflected the common response of

muscle to loss of innervation [16]. According to these findings, we can conclude that SCD1 down-regulation occurred precociously in our SOD1(G86R) mouse model. We then showed here that the decrease in SCD1 expression also persisted during the course of the disease, at 90 days of age, when muscle denervation becomes detectable and motor deficits usually arise, and at about 105 days of age, when hind legs start to be paralysed. At that moment, the decrease in SCD1 expression was also noticeable in the tibialis anterior, which is another muscle in the mouse hind leg displaying less oxidative metabolism than the gastrocnemius (Figure 1A). As a consequence of the repression of muscle SCD1 expression, we observed that the C18:1/C18:0 fatty acid ratio, an index of the desaturation activity of the enzyme [20], was slightly reduced in presymptomatic muscle extracts but significantly diminished at the end stage in both gastrocnemius and tibialis anterior (Figure 1B). It is noteworthy to mention that our previous studies had shown that SOD1(G86R) mice typically exhibit decreased postprandial lipidemia and increased peripheral clearance of lipids, both of which can be ascribed to muscle hypermetabolism [10]. Therefore, an excess of uptake of exogenous lipids in this tissue could mask otherwise earlier and more robust differences in the index of SCD activity.

To obtain independent evidence that SCD1 down-regulation is a typical feature of ALS, we took advantage of our transcriptome database composed of deltoid biopsies from patients with the sporadic form of the disease [12]. The expression of not only SCD1 but also SCD5, a primate-specific enzyme variant with identical function [21], was lower in ALS patients, as compared to normal control subjects. Furthermore, the repression of SCD1 expression was much more remarkable in a muscle not clinically or electromyography affected than in a muscle at an advanced stage of pathology, characterized at the clinical level by reduced strength and neurogenic electromyography pattern (Figure 2A). That SCD1 down-regulation could be observed both in presymptomatic SOD1(G86R) mouse muscle and in relatively healthy human ALS muscle prompted us to speculate that such a pattern of expression might not be solely related to the loss of muscle innervation characteristic of the disease. To address this question, we compared SCD1 expression in gastrocnemius submitted to acute denervation, as obtained by cutting and removing several millimeters of the sciatic nerve, or transient denervation followed by re-innervation, as obtained by crushing the sciatic nerve for several seconds. Under these conditions, the expression of SCD1 was increased after axotomy but significantly reduced after crush (Figure 2B). Overall, these findings provide evidence for the implication of SCD1 in the pathological process triggering ALS,

and suggest that SCD1 down-regulation could be involved in the restoration of muscle function in response to injury.

SCD1 knockout mice do not manifest motor impairment but display exacerbated muscle metabolic oxidative capacity

To gain insight into the way in which the lack of SCD1 expression impacts on muscle function, we investigated several characteristics of muscles in these SCD1 knockout mice reflecting their metabolic status, and also evaluated their motor behavior. At the molecular level, we measured the expression of PGC1- α , PPAR α and PDK4, of which an increase is involved in stimulating mitochondrial biogenesis and in switching the energy source from glucose to fatty acids [22]. The expression of these genes was significantly higher in the gastrocnemius of SCD1 knockout mice, as compared to wild-type littermates; in the tibialis anterior, there was also a trend toward an increased expression (Figure 3A). Despite this latter attenuated response, the tibialis anterior represents, better than the gastrocnemius, a typical example of glycolytic muscle in which to evaluate changes in the relative density of the various fiber types. We therefore used this muscle to determine potential morphological and biochemical changes triggered by the absence of SCD1. The number of fibers per muscle section was higher in SCD1 knockout mice than in wild-type mice (Figure 3B). Accordingly, the distribution of fiber calibers showed an increase in the amount of fibers of small caliber in SCD1 knockout mice (Figure 3C). We extended these findings by performing SDH histochemistry, and found that the average cross-sectional area of both SDH-positive and SDH-negative fibers was smaller in SCD1 knockout mice than in wild-type mice (Figure 3D). These differences were associated in SCD1 knockout mice with a significant predominance of SDH-positive fibers, which are characterized by a higher metabolic oxidative capacity (Figure 3E).

Evaluation of muscle function using the grip strength test revealed no changes in the force developed by hind limbs between SCD1 knockout mice and their wild-type littermates (0.37 ± 0.024 N in SCD1 knockout mice *versus* 0.37 ± 0.021 N in wild-type mice, $n = 7$). Along with this, no abnormal spontaneous electrical activity, which would have reflected the typical response of muscle to loss of innervation, was found in the gastrocnemius of SCD1 knockout mice (data not shown). In contrast, we also measured the expression of a series of genes specific to the motor end plate, including the acetylcholine receptor subunits α , γ and ϵ (AChR- α , AChR- γ and AChR- ϵ , respectively), and muscle-specific receptor tyrosine kinase (MuSK). Except for AChR- γ , of which an increase would have been considered a sign of muscle denervation [23], the expression of these genes was significantly increased in the gastrocnemius of SCD1 knockout mice as compared to their wild-type littermates, although the changes were less pronounced in the tibialis anterior (Figure 4). In all, these results indicate that the genetic ablation of SCD1 is not detrimental *per se* to muscle function but promotes a metabolic shift toward a more oxidative capacity, and stimulates the neuromuscular junction gene expression program.

SCD deficiency accelerates muscle function recovery after nerve injury

As shown above, the lack of SCD1 expression does not represent a handicap for muscle function. Therefore, its down-regulation, as observed in ALS or after nerve crush, prompts us to hypothesize that the enzyme may participate in the restorative efforts that muscles experience at the early stages of disease, when

neuromuscular deterioration is not generalized yet, or during the process of recovery following a brief disruption of the neuromuscular communication. To address this question, we took advantage of such a model of transient denervation and re-innervation as a means to evaluate, by performing relatively manageable short-term experiments [3], the importance of SCD1 for the restoration of muscle function in response to nerve damage. We performed these experiments using not only SCD1 knockout mice but also mice deficient in SCD enzymatic activity, as obtained by feeding them with MF-438, which is an orally bioavailable pharmacological agent inhibiting SCD-dependent desaturation of fatty acids [17]. To verify if our treatment was biologically active *in vivo*, we measured several parameters that should reflect the deficiency in SCD enzymatic activity. First, MF-438 significantly reduced both C16:1/C16:0 and C18:1/C18:0 fatty acid ratios in circulating lipids (Figure 5A). Second, MF-438 also induced a decrease in the respiratory quotient as determined by indirect calorimetry, which indicated a switch of the energy source from glucose to fatty acids (Figure 5B). Finally, the drug triggered concomitantly a small but significant decrease in body mass of mice treated for two weeks (Figure 5C). SCD deficiency did not alter hind limb grip strength during the 2-week treatment, as determined either by a percentage of peak force at day 0 (Figure 5D) or by normalizing peak force to body mass (Figure 5E). Also, there were no detectable electromyography abnormalities suggestive of denervation (data not shown). Furthermore, MF-438 stimulated the expression of several genes specific to the motor end plate (Figure 5F). Overall, these findings strongly support that the effects of the SCD inhibitor are very similar to those observed in SCD1 knockout mice.

To monitor the recovery of muscle function after crushing the sciatic nerve, we measured hind limb grip strength during a post-lesion period of two weeks, and found that the force in SCD1 knockout mice was restored to its initial level more rapidly than in their wild-type littermates. Accordingly, the proportion of abnormal electromyography episodes reflecting neurogenic muscle denervation was lower in SCD1 knockout mice at 14 days post-lesion (Figure 6A). We also measured at that time the relative density of muscle fiber types as a witness to the restorative process. In the denervated tibialis anterior of wild-type mice, the proportion of fibers intensely stained by SDH histochemistry (presumably, slow-twitch type I fibers) was very low, and there was a significant predominance of medium-stained fibers (presumed fast-twitch fatigue-resistant type IIA fibers). In contrast, the distribution of fiber types in the denervated tibialis anterior of SCD1 knockout mice was identical to that observed in the muscle contralateral to the lesion (Figure 6B), suggesting the establishment of a normal non-stressed situation. To corroborate these findings, we followed muscle function recovery after crush in MF-438 treated mice, and also found accelerated, though not complete, restoration of grip strength, as well as reduced extent of electromyography abnormalities (Figure 6C). Furthermore, quantification of the percentage of mice that, after initial total paralysis, started to exhibit a grip strength distinct from zero showed that on average the recovery in treated mice took place three days sooner than in untreated mice (Figure 6D). In all, these results strongly suggest that reducing SCD enzymatic activity stimulates the restorative potential of skeletal muscles.

Discussion

SCD1 is an essential lipogenic enzyme thought to be implicated in the development of obesity and associated metabolic disorders [24]. In this study, we have shown that its expression is down-regulated in skeletal muscles suffering from slowly progressing

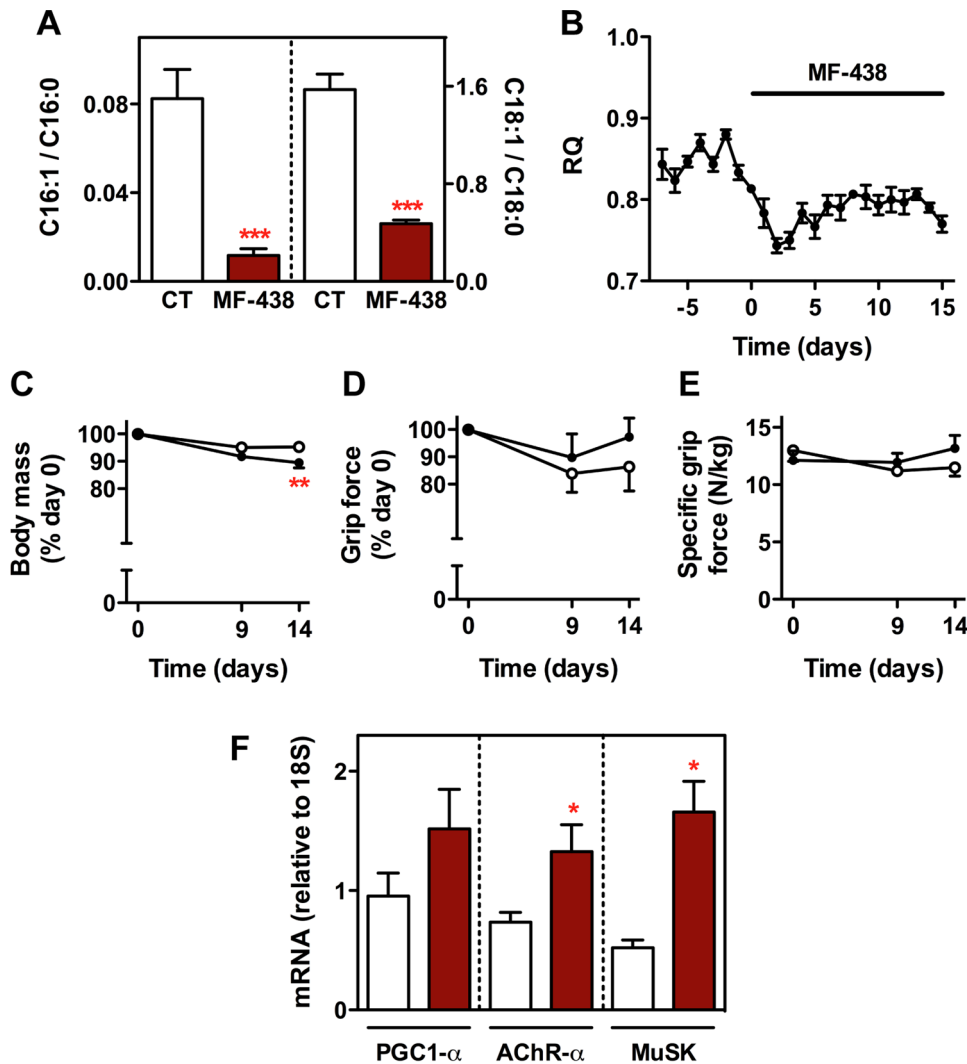


Figure 5. Effects of MF-438 on metabolism and muscle function. (A) C16:1/C16:0 and C18:1/C18:0 fatty acid ratio in plasma from MF-438 treated mice (brown columns) and control littermates (CT, white columns). *** $P < 0.001$ (Unpaired t-test, $n = 4-6$). (B) Time course of respiratory quotient (RQ) before and after treatment with MF-438 at a dose of 10 mg/kg body mass/day (indicated by the black bar) ($n = 4$). Time course of body mass (C), muscle grip strength expressed as a percentage of day 0 (D), and specific grip strength, as determined by normalizing peak force to body mass (E), in mice fed regular chow (white circles) and mice fed regular chow supplemented with MF-438 (black circles). ** $P < 0.01$ (2-way ANOVA followed by Bonferroni test, $n = 5-6$). (F) Expression of PGC1- α , AChR- α , and MuSK in gastrocnemius from MF-438 treated mice (brown columns) and control littermates (white columns). * $P < 0.05$ (Unpaired t-test, $n = 3-9$). doi:10.1371/journal.pone.0064525.g005

motor neuron degeneration, as seen in ALS, and from transitory denervation/re-innervation, as obtained experimentally by sciatic nerve crush. Based on these findings, we have also shown here that the systemic down-regulation of SCD1, as generated by both genetic and pharmacological means, enhances the oxidative metabolism of muscles, stimulates the expression of synaptic genes, and ameliorates the restoration of muscle function following transient denervation and subsequent re-innervation.

SCD1 knockout mice are mainly characterized by decreased adiposity and increased metabolic rate [14], two characteristics of the mutant SOD1 mouse model of ALS, even observed before the onset of any motor neuropathology [9]. The lack of SCD1 expression has been associated with inhibition of lipogenesis and stimulation of mitochondrial β -oxidation of fatty acids [25]. Notably, we had previously observed that the expression of genes involved in the uptake of fatty acids, such as FAT/CD36 and other related genes, was presymptomatically increased in skeletal

muscle of SOD1(G86R) mice [10]. In addition, it is known that the muscle-specific overexpression of FAT/CD36 is sufficient to trigger fatty acid oxidation [26]. It seems therefore not very surprising that, in accordance with an enhanced uptake of fatty acids, the muscles of SOD1(G86R) mice exhibit concomitantly a decrease in the expression of SCD1. The situation appears to be less straight-forward in the case of ALS patients. Although most of them are markedly hypermetabolic [6], as in the animal model, they usually present with increased levels of circulating lipids [7], which would not intuitively substantiate the hypothesis of the muscle down-regulation of SCD1. It is noteworthy, however, that the expression of SCD1 in patients was much more repressed in muscles not clinically or electromyography affected, which lets envisage that SCD1 down-regulation might occur as a result of whatever mechanism preceding overt disease. The fact that SCD1 down-regulation was observed early in presymptomatic SOD1(G86R) mouse gastrocnemius and in relatively healthy

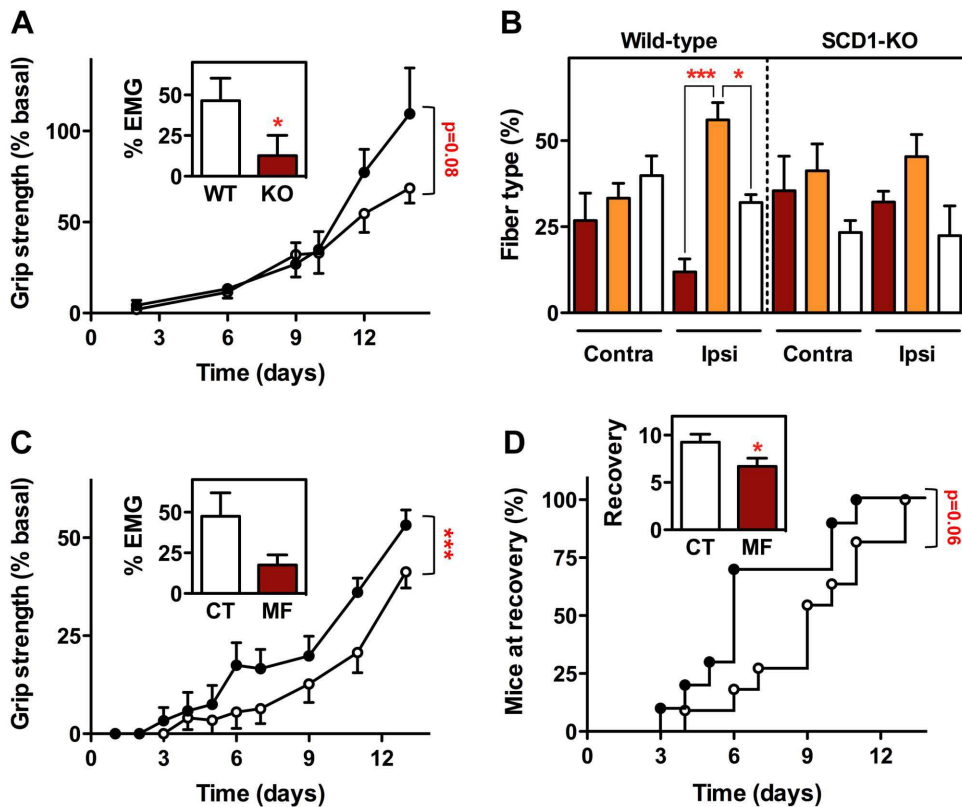


Figure 6. Muscle function recovery in SCD-deficient mice submitted to nerve crush. Restoration of muscle grip strength in SCD1 knockout mice (A) or MF-438 treated mice (C) (black circles) and corresponding control littermates (white circles) at the indicated post-operation times. $***P < 0.001$ (2-way ANOVA, $n = 4-12$). Percentage of electromyography episodes of spontaneous activity in SCD1 knockout mice (Inset A) or MF-438 treated mice (Inset C) (KO or MF, brown columns) and corresponding control littermates (WT or CT, white columns) two weeks after sciatic nerve crush. $*P < 0.05$ (Unpaired t-test, $n = 4-7$). (B) Relative density of muscle fiber types in ipsilateral and contralateral tibialis anterior from SCD1 knockout mice and wild-type littermates two weeks after sciatic nerve crush. According to SDH histochemistry, fibers were classified as dark brown colored fibers with high metabolic oxidative capacity (brown columns), pale brown colored fibers with medium oxidative capacity (orange columns) and non-saturated fibers (white columns). $*P < 0.05$ and $***P < 0.001$ (1-way ANOVA followed by Tukey's multiple comparison test, $n = 4-6$). (D) Kaplan-Meier curves showing the percentage of MF-438 treated mice (black circles) and control littermates (white circles) that started to exhibit a grip strength distinct from zero after initial total paralysis. Logrank test ($n = 10-11$). Inset D, averaged time at start of recovery in MF-438 treated mice (MF, brown column) and control littermates (CT, white column). $*P < 0.05$ (Unpaired t-test, $n = 10-11$). doi:10.1371/journal.pone.0064525.g006

human ALS deltoid suggests that the transcriptional regulation of SCD1 might not be (solely) related to the loss of innervation of muscles characteristic of these conditions. In support of this notion, such a down-regulation of SCD1 expression was not found after severe denervation, at least at a time when re-innervation was not present according to our protocol of axotomy. In contrast, a more subtle denervation, as that obtained by crushing the sciatic nerve for only several seconds, simulated the inhibitory effect of ALS on SCD1 expression. Because sciatic nerve crush allows the rapid recovery (in around two weeks) of muscle function, we speculate that SCD1 down-regulation may be somehow related to the restorative potential of skeletal muscles.

As a first step toward the understanding of the importance of SCD1 for muscle function, we found that the genetic ablation of SCD1 promoted a decrease in the cross-sectional area of muscle fibers, and an increase in the amount of those enriched in the mitochondrial enzyme SDH. In parallel, we also observed increased expression of PGC1- α , PPAR α and PDK4. Overall, these observations provide persuasive evidence for a higher metabolic oxidative capacity, in accordance with several previous studies. For instance, overexpressing PGC1- α has been reported to trigger a decrease in the size of muscle fibers and a concomitant

fast to slow fiber type shift [27]. In contrast, knocking out PGC1- α specifically in muscle has been shown to induce a shift from oxidative to glycolytic muscle fibers [28]. Moreover, PGC1- α not only triggers metabolic changes in muscle but also activates the expression of genes specific to the motor end plate [29]. Therefore, the increased expression of the signaling kinase MuSK and several acetylcholine receptor subunits that we observed in our SCD1 knockout mice could be the consequence of a higher transcriptional activity involving, at least in part, PGC1- α . Despite these modifications, the lack of SCD1 did not cause any alteration of muscle function as determined by behavioral and electrophysiological means. Furthermore, inhibiting global SCD enzymatic activity with a diet containing MF-438 induced very similar changes as those found in SCD1 knockout mice.

Subsequent efforts were concentrated in determining if SCD1 down-regulation might affect muscle function when challenged by sciatic nerve crush. Both SCD1 knockout mice and MF-438 treated mice recovered their force more rapidly than their corresponding control groups. Two weeks after lesion, the degree of denervation was more important in these control groups than in SCD-deficient mice. In support of these findings, very early studies had already reported that oxidative muscles can recuperate better

than glycolytic ones after nerve crush [30]. Also, PGC1- α has been shown to protect muscles from denervation atrophy [31]. It can be therefore postulated that the pro-restorative power of SCD deficiency is the result of an enhancement of oxidative metabolism, as that shown in this work. A fast to slow fiber type shift has been shown to occur progressively in ALS, and it has been proposed that this metabolic modification would render muscle fibers with higher oxidative capacity more resistant to disease [32]. Recent studies have further reported that a modest increase in the expression of MuSK, as that observed here, can maintain neuromuscular junctions in mutant SOD1 mice, hence retarding denervation and ameliorating muscle function [33]. Taking our present findings as a whole, we can put forward that SCD1 stands at a regulatory crossroad shaping muscle function in health and disease. In this respect, the beneficial effects of inhibitors of SCD

enzymatic activity could pave the way for developing novel therapeutic strategies to palliate motor neuron injury.

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Author Contributions

Conceived and designed the experiments: GH FS JLGDA JPL. Performed the experiments: GH FS AH TL FR SDG LP. Analyzed the data: GH FS AH TL FB SDG HO LD EM JLGDA JPL. Contributed reagents/materials/analysis tools: FB HO MHMB EM. Wrote the paper: JLGDA.

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Chapter 2:

Fatty acids profiling of mSOD1 mice

The fatty acid ratios have been used as biomarkers for functions and dysfunctions of various systems, enzymatic activities, and cellular processes in the human body. Recently, it is an emerging idea of measuring fatty acid ratios in various tissues as a prediction of onset or stage of diseases, simply speaking “biomarkers”. Mostly all of the neurodegenerative diseases display an alteration in lipids metabolism and energy homeostasis even prior to the onset of clinical symptoms, therefore product to substrate ratio of fatty acids in such conditions may help to follow the disease at a very early stage. Predominantly, the ratios have been used to assess the fatty acid intake in relation to the dietary intake for assessing the nature and effect of particular dietary fatty acids on metabolism, storage, and influence on human health (Garaulet et al., 2001; Knutsen et al., 2003; Hodson et al., 2008). Normally, gas chromatography (GC) or high performance liquid chromatography (HPLC) accompanied with mass spectrometry is used to measure MUFAs, PUFAs, NEFAs, TAG and other lipid fractions in plasma, serum, adipose tissues, liver, brain, spinal cord, and muscles (Evans et al., 2002; Seppänen-Laakso et al., 2002; Baylin et al., 2005; Menéndez-Carreño et al., 2008; Hodson et al., 2009; Klepacki et al., 2012).

The comparison of MUFAs from various source adipose tissues has been reported to be linked to cardiovascular diseases for example; gluteofemoral adipose tissues have higher levels of 16:1 n-7 and SCD1 mRNA as compared to abdominal subcutaneous adipose tissues (Pinnick et al., 2012). Similarly, the prevalence of linoleic acid (C18:2 n-6) in adipose tissues had been documented in negative correlation with cardiovascular disease risk (Riemersma et al., 1986). The concentration and tissue ratio of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been suggested as markers of coronary heart disease (Harris et al., 2007). The abundance of very long-chain omega-3 fatty acids (VLC n-3) and stearic acid in serum free fatty acid (FFA) has been reported to be inversely associated with myocardial infarction risk and it is suggested that these ratios of VLC n-3 and free fatty acids in one way or other might be related to the pathogenic process of this disease (Yli-Jama et al., 2002). The plasma fatty acid profile has been compared in the patients with liver cirrhosis or acute myeloid leukemia to healthy subjects and it has been found altered in diseased persons (Patterson et al., 2011). It is suggested that the measurement of oxysterol, cholesterol metabolites, in plasma can be used as a marker of AD in

patients with cardiovascular disease (Hughes et al., 2012). Very closely resembling results for plasma MUFAs ratio as a marker of various diseases have also been reported (Lands, 1995; Browne and Armstrong, 2000; Arab and Akbar, 2006). The lipids profiling utility is not limited as biomarkers of neurodegenerative diseases, it is used to analyse the food intake and patterns of daily nutritional value (Arab, 2003; Poppitt et al., 2005; Baylin and Campos, 2006; Saadatian-elahi et al., 2009).

The ratio of product unsaturated fatty acids to substrate saturated fatty acids has been widely used in various studies to investigate the enzymatic activity of SCD1 in fatty acid metabolism and in a number of diseases. In one study, liver specific SCD1 inhibitor was used and a ratio of oleic acid to stearic acid in plasma was measured as an indication of SCD1 activity (Landry et al., 2011). The expression of hepatic SCD1 mRNA is strongly correlated with the product to precursor ratio (16:1/16:0 and 18:1/18:0) of liver lipid fractions and similarly the ratio of 16:1/16:0 and 18:1/18:0 in plasma VLDL was found as an indicator of SCD1 expression in liver (Peter et al., 2009).

Taking together, it is well supported from the above mentioned studies that lipids profiling can be a reliable and authenticated approach to be used as a biomarker of various maladies. The product to the precursor ratio of particular fatty acids and measurement of metabolites of specific lipids in various tissues for example brain, spinal cord, plasma, serum, liver, muscle and adipose tissues can reflect any alteration going on in these tissues.

In the previous chapter we have illustrated altered 16:0/16:1 and 18:0/18:1 ratio in the muscle of mSOD1 arising from the decrease of SCD1 expression. It has already been well elaborated that these changes in SCD1 mRNA expression occur even at pre-symptomatic stage in mSOD1 mice suggesting an important role of these lipid fractions in diseases as causative, progressive or protective elements of pathological conditions in course of disease. Lipids are not limited to these a few fatty acids rather this a monster class comprising SFAs, MUFAs, PUFAs and numerous others biologically active molecules with the persistent presence in both simple and complex organisms. Therefore it seems to be pregnant with plenty of information to explore lipid fractions other than mentioned above. In the current part of our study, we made an effort to work

out the alterations in all fatty acids whether simple or complex by using GC to highlight their participation in fatal neurodegenerative disorder like ALS. We performed our analyses on samples from serum and liver of mSOD1 mice and control littermates of same age.

**Systemic reduction of stearyl-CoA desaturase activity
occurs at onset of muscular denervation
in an animal model of amyotrophic lateral sclerosis**

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular degenerative disease, associated with important metabolic alterations at the whole body level, including hypermetabolism and dyslipidemia. Our previous studies, performed on mice and humans with ALS, revealed changes in the muscle expression of stearoyl-CoA desaturase-1 (SCD1), an enzyme that introduces the first *cis* double bond in saturated fatty acid substrates. On the basis of this finding, we analyzed here the composition of fatty acids in neutral lipids (*e.g.*, triacylglycerides and steryl esters) in blood and liver from mutant SOD1 mice, a model of ALS. Then, we correlated the observed changes with the onset and progression of disease in these mice. We found reduced relative SCD enzymatic activity in blood and liver, as estimated by the oleic acid/stearic acid ratio, which coincided with the onset of subtle, subclinical muscular denervation. This result was further reinforced by significant decreases in the proportion of oleic acid in blood and liver of overtly symptomatic animals, in association with diminished concentrations of circulating triglycerides and total cholesterol. We also showed that pharmacological inhibition of SCD enzymatic activity, as obtained by treating naive mice with MF-438, reproduced the same trait of disturbances in fatty acid composition and circulating levels of neutral lipids. In all, our findings strongly suggest that SCD1 is instrumental in establishing the metabolic phenotype of ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a degenerative disease characterized by progressive loss of upper and lower motor neurons. As a result of neurodegeneration, patients present with progressive muscular weakness evolving toward atrophy and paralysis. The first symptoms appear in adulthood between 40 and 70 years of age, and death occurs 2 to 5 years after diagnosis. Studies conducted on genetic animal models of ALS showed that the process leading to motor neuron degeneration is not cell-autonomous but involves defects in other cell types than neurons [1-4]. Consistent with this notion, ALS neurodegeneration is associated with systemic defects, including hypermetabolism and dyslipidemia, which are observed in both patients and animal models [5-7]. Particularly, transgenic mice overexpressing a mutated form of Cu/Zn superoxide dismutase (SOD1), linked to familial ALS, are in energy deficit and have decreased adipose tissue stores. These deficiencies appear to be elicited by increased energy expenditure, and due to an increased consumption of nutrients by skeletal muscles [8,9].

To gain insight into the relationships between neurodegeneration and metabolic dysfunction, we previously analyzed the gene expression profiles of skeletal muscles from mutant SOD1 mice and patients with ALS [10,11]. We found a decrease in the expression of stearoyl-CoA desaturase-1 (SCD1), an enzyme that introduces the first *cis* double bond in the delta-9 position of saturated fatty acid (SFA) substrates. The preferred substrates are palmitic acid (C16:0) and stearic acid (C18:0), which are converted to palmitoleic acid (C16:1) and oleic acid (C18:1), respectively [12]. These monounsaturated fatty acids (MUFAs) are the major constituents of complex lipids such as diacylglycerols, phospholipids, triglycerides, wax esters and cholesterol esters. Recently, we also showed that repressing SCD1 expression or reducing SCD-dependent enzymatic activity enhances the oxidative metabolism of muscles, stimulates the expression of synaptic genes, and ameliorates motor restoration following peripheral nerve injury [13].

Fatty acids are of paramount importance to all cells, since they provide energy, function as signaling molecules, and sustain structural integrity of cellular membranes. In the nervous system, where fatty acids are found in huge amounts, they participate in its development and maintenance throughout life. Growing evidence strongly indicates that fatty acids in their own right are also implicated in pathological conditions, including neurodegenerative diseases, mental disorders, stroke and trauma [14]. In this study, we analyzed the composition of fatty acids in neutral lipids (*e.g.*, triacylglycerides and steryl

esters) in blood and liver from mutant SOD1 mice, as determined by gas chromatography. We also correlated the observed changes with the onset and progression of disease in these mice. We conclude that SCD1 is instrumental in establishing the metabolic phenotype of ALS.

METHODS

Animals

FVB/N mice overexpressing the murine G86R SOD1 mutation (SOD-1 mice) [15], were maintained in our animal facility at 23°C with a 12 hours light/dark cycle. They had water and regular A04 rodent chow ad libitum. Age-matched non-transgenic female littermates served as controls. SCD activity was inhibited with the pharmacological agent MF-438, or 3-(5-methyl-[1,3,4]thiadiazol-2-yl)-6-[4-(2-trifluoromethyl-phenoxy)-piperidin-1-yl]-pyridazine (Prestwick Chemical, Illkirch, France). FVB/N mice were fed with A04 chow ad libitum containing 0.00625% (w/w) MF-438 (Safe, Augy, France), which provided a daily dose of 10 mg/kg body mass, as calculated on a basis of 4 g of food intake per day and 25 g of average body mass. Age-matched littermates fed on regular A04 chow pellet (Safe, Augy, France) served as controls. Animal experiments followed European Union regulations (Directive 2010/63/EU), were approved by the ethical committee of the University of Strasbourg (No. AL/01/20/09/12) and performed by authorized experimenters (No. A67-402).

Sample collection

After deep anesthesia with sodium pentobarbital (120 mg/kg, Ceva Santé Animale, France), electromyograms were obtained with a standard electromyography apparatus (Dantec, Les Ulis, France), in accordance with the guidelines of the American Association of Electrodiagnostic Medicine. A concentric needle electrode (no. 9013S0011, diameter 0.3 mm; Medtronic, Minneapolis, MN) was inserted into the gastrocnemius, and a monopolar needle electrode (no. 9013R0312, diameter 0.3 mm; Medtronic) was inserted into the tail of the mouse to ground the system. Only spontaneous activity with at least 50 μ V of amplitude was considered. Right after, whole blood was collected in plain tubes and mice were transcardially perfused with ice-cold PBS. Liver was rapidly dissected and snap frozen in liquid nitrogen. Whole blood was centrifuged at 4000 rpm, for 4 minutes, at 4°C, serum was collected and snap frozen. Samples were kept at -80°C until further steps.

Neutral lipid extraction

Lipids from the serum were extracted following a modified version of the Bligh and Dyer method [16]. Briefly, methanol/chloroform solution was added to the serum.

Centrifugations were performed after the addition of chloroform and KCl. The lower phases were collected and mixed with methanol. After a second centrifugation, the lower phase containing the lipids was collected in glass tubes. The liver samples were weighed, and disrupted with Tissue Lyser twice at 30 Hz for 3 min each by using stainless steel beads and racks precooled at -80°C. After removing the beads, 1 mL ice-cold Ripa buffer was added per 100 mg of disrupted tissue, and samples were centrifuged at 2000 g for 10 min at 4°C. A volume of supernatant corresponding to 2 mg of proteins, according to the bicinchoninic acid assay, was mixed with 2.5 mL chloroform/methanol (1:1). After vortex and sonication, the samples were incubated for at least 2-3 hours at 4°C, and centrifuged at 2000 g for 10 min at 4°C. Supernatants were collected and non-polar lipid fraction was obtained by separation on Sephadex columns. Neutral lipids were transmethylated with methanol and potassium hydroxide, and collected in heptane. The fatty acid methyl esters were stored at -20°C until further steps.

Gas chromatography

Gas chromatography was performed with 1 µL of fatty acid methyl esters, using a Varian 3400 CX chromatograph fitted with a WCOT fused silica capillary column of 100 m x 0.25 mm x 0.20 µm (coated with polar highly substituted cyanopropyl CP-SIL 88 phase). The temperature gradient in the oven ranged from 80 to 220°C at a rate of 4°C/min. We used helium as gas carrier. The temperature of the flame ionization detector was set at 270°C. The peaks were identified by retention time and compared to a standard mix of fatty acid methyl esters (Supelco 37 and Supelco PUFA-2 Animal Source; Sigma-Aldrich, Saint-Quentin Fallavier, France). Data were expressed as relative percentages. An index of relative SCD enzymatic activity was estimated by the 18:1/18:0 ratio [17].

Direct quantification of lipids

Levels of triglycerides and total cholesterol were determined with the Randox kits TR210 and CH200, respectively, according to the manufacturer's recommendations. Briefly, the TR210 kit is based on the hydrolysis of triglycerides to release glycerol that reacts with H₂O₂. Subsequent enzymatic reactions produce a colored end-product. Absorbance is measured at 550nm. CH200 kit is based upon the oxidation of cholesterol to cholestene 3-one and H₂O₂ by the cholesterol oxidase. The H₂O₂ produced is then dosed upon the same principle as with the TR210 kit.

Real-time RT-PCR

Total RNA was prepared following standard protocols. Briefly, frozen liver was placed into a tube containing a 5-mm stainless steel bead. Working on ice, 1 mL Trizol reagent (Invitrogen, Groningen, The Netherlands) was added, and homogenisation was performed in a Tissue Lyser (Qiagen, Valencia, CA) at 30 Hz for 3 min twice. RNA was extracted with chloroform/isopropyl alcohol/ethanol and stored at -80°C until use. One μg of total RNA was used to synthesize cDNA using Iscript reverse transcriptase (BioRad Laboratories, Marnes La Coquette, France) and oligo-dT primer as specified by the manufacturer. Gene expression was measured using the SYBR green reagent (2x SYBR Green Supermix; Bio-Rad Laboratories) following the manufacturer's instructions on a Bio-Rad iCycler. PCR was performed in optimized conditions: 95°C denatured for 3 min, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C . Primers (Eurogentec, Seraing, Belgium) were as follows: RNA polymerase II (pol2): forward 5'-aatccgcatcatgaacagtg-3', reverse 5'-tcattcattttatccaccacc-3'; TBP: forward 5'-ccaatgactcctatgacccta-3', reverse 5'-cagccaagattcagcgtagat -3'; SCD1: forward 5'-cctacgacaagaacattcaatcc-3', reverse 5'-cgtctcaagttctttaatcct-3. Relative quantification was achieved by calculating the ratio between the cycle number (Ct) at which the signal crossed a threshold set within the logarithmic phase of the gene of interest and that of the normalization factor determined by GeNorm with the three reference genes (pol2 and TBP). Ct values were the means of duplicates.

Statistics

Data are expressed as the mean \pm SEM. Prism version 5.0a software (GraphPad, San Diego, CA) was used for statistical analysis. Statistics were performed using unpaired t-test or one way ANOVA. A p-value of less than 0.05 was considered significant.

RESULTS

Altered fatty acid profile in the serum of SOD1 mice

We have previously shown that ALS patients and SOD1 mice present with systemic metabolic dysfunction that impacts on disease severity [6,8]. Here, we pictured the profile of circulating fatty acids in SOD1 mice and wild-type littermates. After extraction of total lipids and their separation into polar and non-polar fractions, the latter further underwent transmethylation, allowing fatty acids present in the form of triglycerides and steryl esters to be released and identified by gas chromatography. On the basis of the carbon chain length and the number of double bonds, fifteen fatty acids were identified, ranging from palmitic acid (16:0) to docosahexaenoic acid (22:6 n-3). **Table 1** shows the proportions of each fatty acid at pre-symptomatic and symptomatic stages. When SOD1 mice do not exhibit any pathological sign, the profile of circulating fatty acids was normal and the balance between SFAs, MUFAs and polyunsaturated fatty acids (PUFAs) remained unchanged. An altered fatty acid profile appeared when mice, with or without overt motor impairment, already showed abnormal electromyography recordings indicative of denervation-induced spontaneous electrical activity. At this stage, the proportion of MUFAs diminished, whereas PUFAs appeared over-represented (**Table 1**). Examples of altered fatty acids were oleic acid (18:1), arachidonic acid (20:4 n-6) and docosahexaenoic acid (22:6 n-3).

Hepatic fatty acids

Liver is an organ of the utmost importance to the regulation of lipid metabolism, and hence determines considerably the composition of circulating fatty acids. In order to explore the implication of liver in the observed alterations in serum, we also investigated the hepatic pool of fatty acids (**Table 1**). Their proportions were quite stable in the liver of pre-symptomatic mice, except for a significant under-representation of the essential alpha-linoleic acid (18:3 n-3). We also noted a trend for higher levels of SFAs in SOD1 mice, which became significant at symptomatic stage. During this phase, the most representative SFAs and MUFAs were respectively over- and under-represented, suggesting a reduced production of MUFAs from SFAs.

Relative SCD enzymatic activity in SOD1 mice

The rearrangement of the pool of fatty acids that we observed in SOD1 mice can reflect important alterations of lipid biosynthetic pathways associated with the progression of disease. To address this question, we studied more in detail the potential correlation between the observed changes in fatty acid composition and different (sub)phases of the pathological process. In fact, the progression of symptoms in SOD1 mice can be easily

followed according to the extent of the variations in the spontaneous electrical activity displayed by denervated muscles. In our hands, a long pre-symptomatic stage was characterized by the absence of both apparent, clinically relevant motor abnormalities and a silent electromyographic signal (**Figure 1B**). Then, muscles of mice at the beginning of the symptomatic stage started to develop subtle spontaneous electrical activity variations occurring without noticeable motor impairment (**Figure 1C**). Finally, late symptomatic mice clearly showed impaired hind limb extension reflexes, gait impairment and onset on paralysis in association with severe denervation (**Figure 1D**). In accordance with this classification, relative SCD enzymatic activity, as estimated by the oleic acid (18:1)/stearic acid (18:0) ratio, was found to be reduced in serum and liver of both early and late symptomatic mice (**Figure 2A**). Along with this finding, the expression of SCD1 at mRNA and protein level was also lower during the symptomatic stage, irrespective of the extent of denervation (**Figure 2B**). Taken together, these results show a loss of SCD activity that correlates with the onset of denervation in an animal model of ALS.

Pharmacological modulation of SCD enzymatic activity

SCD1 is a key enzyme in the regulation of lipid metabolism, and its activity influences fatty acid beta-oxidation and insulin sensitivity [18]. To evaluate whether altered SCD enzymatic activity contributes to overall dysfunction of energy metabolism in SOD1 mice, we treated normal mice with MF-438, which is an orally bioavailable inhibitor of SCD enzymatic activity. After seven days of treatment, the proportions of fatty acids resembled those observed in SOD1 mice (**Table 2**). Indeed, MF-438 was shown to decrease SCD activity index (**Figure 3A**), and body mass (**Figure 3B**), despite normal food intake (**Figure 3C**). Moreover, circulating levels of triglycerides (**Figure 4A**) and total cholesterol (**Figure 4B**) were reduced both in SOD1 mice, as expected, and in MF-438 treated mice. Taken together, these data argue for a prominent role of SCD1 in establishing altered energy metabolism in SOD1 mice.

DISCUSSION

In the present study, we analyzed the composition of peripheral fatty acids at different stages of disease in an animal model of ALS. First biochemical changes manifested in mice at the onset of modest, subclinical muscular denervation, and they mainly concerned a reduction in the synthesis of MUFAs by SCD enzymatic activity. This phenomenon was still noticeable at more severe stages of disease, characterized by overt denervation and motor impairment. High SCD1 activity has been associated with synthesis of fatty reserves and insulin resistance [19][20], whereas low SCD1 activity has been shown to be responsible for enhanced lipid catabolism [21]. The expression of SCD1 is tightly regulated by food intake and levels of circulating fatty acids. An excess of SFAs, carbohydrates and cholesterol up-regulate its expression [22]. Chronic food restriction and subsequent refeeding, which is known to promote fat storage, also causes SCD1 up-regulation. Conversely, long term fasting is associated with a down-regulation of hepatic SCD1 [22]. In the present study, the loss of SCD enzymatic activity in liver does not seem to be triggered by fasting, as SOD1 mice exhibit normal food intake, at least, during the early phases of symptomatic disease [8]. Alternatively, it has been also documented that SCD1 is down-regulated by increased levels of PUFAs. Of note, PUFA levels appeared augmented in the fatty acid profile of both serum and liver of SOD1 mice.

The negative transcriptional regulation of SCD1 that we observed in liver strongly indicates that *de novo* lipogenesis is reduced in symptomatic SOD1 mice. In addition, the pharmacological inhibition of SCD enzymatic activity by MF-438 also suggests that a low SCD activity could cause some of the lipid alterations found in SOD1 mice, including hypolipidemia. Our previous studies have reported a precocious down-regulation of SCD1 in muscles of SOD1 mice and in a subset of ALS patients [11]. Indeed, at the time of biopsy, the muscles from this subset of patients were spared from detectable electromyography abnormalities. Also, we have recently reported that reducing SCD1 activity, by genetic or pharmacological means, turned skeletal muscles to a more oxidative metabolism, promoted the expression of neuromuscular junction genes and supported peripheral nerve regeneration after sciatic nerve crush [13]. Down-regulation of SCD1 and changes in hepatic metabolism could be therefore a potential intrinsic mechanism to counteract ALS.

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% %	Serum				Liver			
	Control	Presympt	Control	Sympt	Control	Presympt	Control	Sympt
16:0	20.0 ± 0.3	20.5 ± 0.9	20.7 ± 0.7	17.4 ± 1.3 ⁰	24.2 ± 1.7	26.8 ± 1.5	22.4 ± 1.2	27.1 ± 1.6 ¹
18:0	2.7 ± 0.1	2.5 ± 0.2	2.8 ± 0.2	3.2 ± 0.3	4.5 ± 0.8	4.4 ± 0.6	3.5 ± 0.3	5.9 ± 0.8 ¹
20:0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1 ⁰
22:0	0.3 ± 0.0	0.7 ± 0.2	1.2 ± 0.8	2.6 ± 1.7	0.7 ± 0.3	1.2 ± 0.6	0.6 ± 0.2	0.5 ± 0.2
16:1n9	3.4 ± 0.2	3.7 ± 0.4	3.1 ± 0.2	2.1 ± 0.4 ¹	3.5 ± 0.4	3.2 ± 0.3	3.6 ± 0.2	2.6 ± 0.3 ¹
18:1n9	35.8 ± 1.4	33.9 ± 1.6	31.9 ± 1.1	26.4 ± 1.4 ¹	38.6 ± 1.7	38.0 ± 1.4	40.2 ± 1.3	30.3 ± 2.5 ²
20:1n9	0.6 ± 0.1	0.5 ± 0.0	0.4 ± 0.1	0.8 ± 0.3	1.4 ± 0.3	1.0 ± 0.3	1.4 ± 0.3	1.1 ± 0.3
18:2n6	27.2 ± 1.0	25.5 ± 1.1	28.0 ± 0.9	27.6 ± 1.9	20.2 ± 2.2	20.2 ± 0.7	19.3 ± 0.8	22.8 ± 1.5 ⁰
18:3n6	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.0 ⁰	0.9 ± 0.2	0.7 ± 0.1
20:2n6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
20:3n6	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.2	0.4 ± 0.1 ⁰	0.8 ± 0.1	1.3 ± 0.4
20:4n6	5.4 ± 0.4	7.9 ± 1.6	6.7 ± 0.5	13.5 ± 2.5 ¹	1.9 ± 0.3	1.8 ± 0.3	2.9 ± 0.6	3.5 ± 0.4
18:3n3	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	0.6 ± 0.1 ¹	1.2 ± 0.2	1.1 ± 0.3
20:5n3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.8 ± 0.1 ¹	0.5 ± 0.2	0.2 ± 0.0	0.5 ± 0.1	0.4 ± 0.1
22:6n3	2.3 ± 0.3	2.7 ± 0.4	2.4 ± 0.3	3.9 ± 0.5 ¹	0.9 ± 0.2	1.2 ± 0.3	1.6 ± 0.2	1.9 ± 0.3
SFA	22.9 ± 0.4	23.7 ± 0.9	24.7 ± 1.0	23.3 ± 2.1	29.5 ± 1.4	32.5 ± 0.9 ⁰	26.6 ± 1.1	33.8 ± 2.0 ¹
MUFA	39.8 ± 1.6	38.0 ± 1.9	35.4 ± 1.3	29.2 ± 1.5 ¹	43.5 ± 1.8	42.2 ± 1.3	45.1 ± 1.4	34.0 ± 2.5 ²
PUFA	37.3 ± 1.6	38.3 ± 2.6	39.4 ± 1.3	47.5 ± 3.4 ¹	26.4 ± 2.5	25.0 ± 1.0	26.9 ± 1.4	32.1 ± 1.8 ¹

Table 1. Fatty acid composition in serum and liver of SOD1 mice.

Mean values and SEM of fatty acid proportions at two different ages (n=10-15).

0 p<0.1, 1 p<0.05, 2 p<0.005.

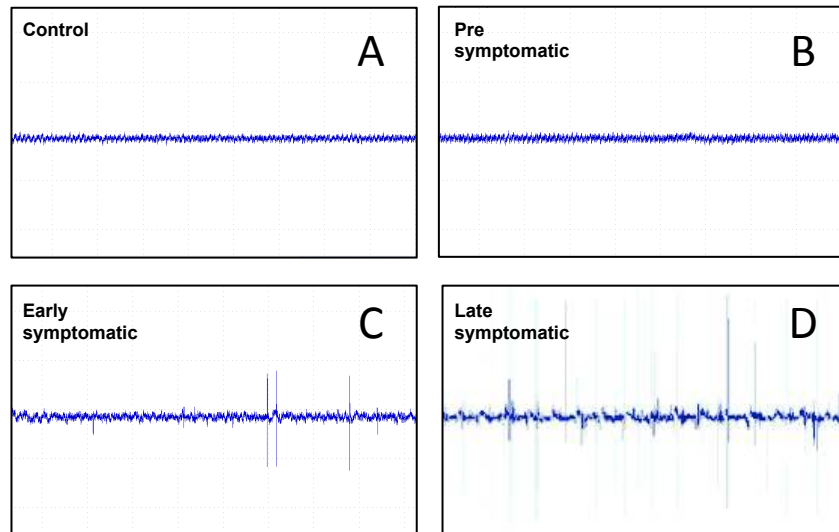


Figure 1. Muscle denervation in SOD1 mice.

Representative electromyograms of spontaneous electrical activity in the different experimental groups included in the study.

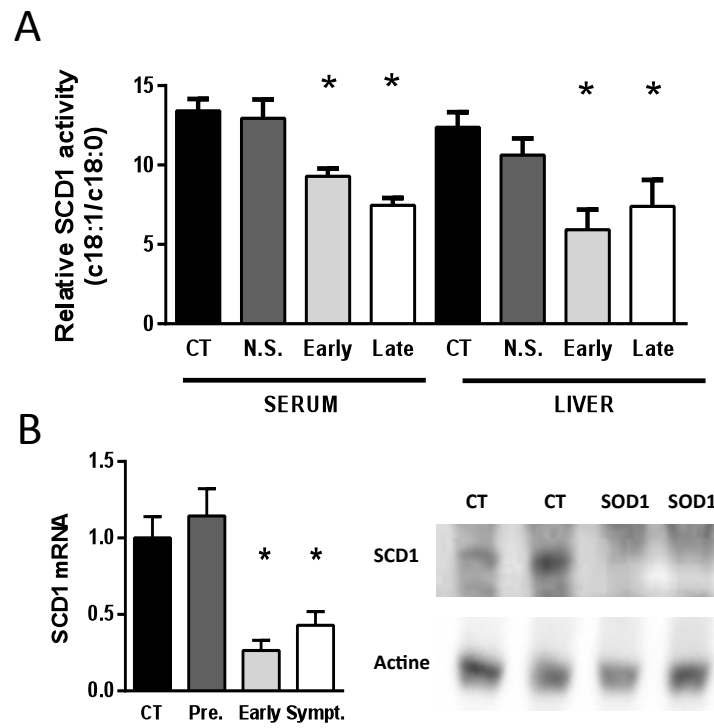


Figure 2. SCD activity and SCD1 expression in SOD1 mice. **(A)** Relative SCD1 enzymatic activity in serum (n=10) and liver (n=15, * p<0.05). **(B)** SCD1 expression at mRNA (left panel; n=6, * p<0.05) and protein level (right panel).

%	Serum - Neutral lipids	
	Control	MF
16:0	18.84 ± 0.84	17.58 ± 1.65
18:0	1.81 ± 0.07	6.74 ± 0.21 ²
20:0	0.02 ± 0.01	0.00 ± 0.00 ⁰
22:0	0.33 ± 0.04	1.17 ± 0.38 ⁰
16:1n9	4.47 ± 0.33	0.45 ± 0.07 ²
18:1n9	29.42 ± 1.53	12.63 ± 0.73 ²
20:1n9	0.59 ± 0.06	0.14 ± 0.05 ²
18:2n6	29.13 ± 1.51	41.39 ± 0.53 ²
18:3n6	0.31 ± 0.04	0.36 ± 0.06
20:2n6	0.25 ± 0.01	0.45 ± 0.13
20:3n6	0.62 ± 0.11	0.39 ± 0.23
20:4n6	8.81 ± 1.12	11.82 ± 1.42
18:3n3	0.97 ± 0.07	1.48 ± 0.31
20:5n3	0.79 ± 0.07	1.68 ± 0.51
22:6n3	3.63 ± 0.32	3.71 ± 0.25
SFA	21.01 ± 0.82	25.49 ± 1.55 ¹
MUFA	34.48 ± 1.87	13.22 ± 0.69 ²
PUFA	44.51 ± 2.52	61.29 ± 2.16 ²

Table 2. Fatty acid composition in serum of mice treated with MF-438.

Mean values and SEM of fatty acid proportions (n=10-15; ⁰ p<0.1, ¹ p<0.05, ² p<0.005).

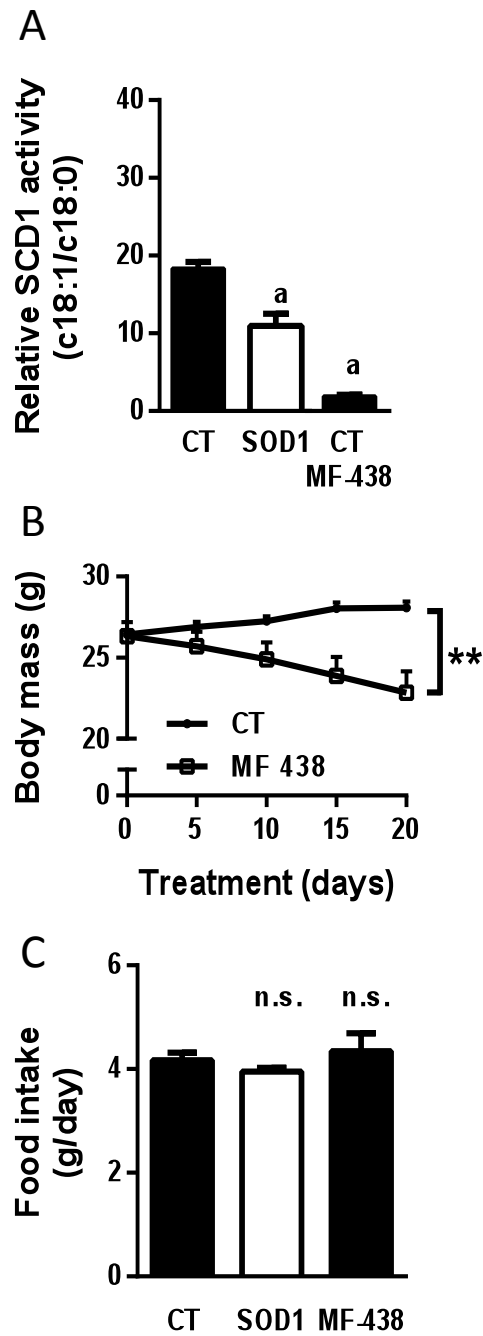


Figure 3. Pharmacological inhibition of SCD activity. Relative SCD enzymatic activity (n=8; a, p<0.05) **(A)**, body mass **(B)** and food intake (n= 8, p<0.05) **(C)** upon MF-438 treatment.

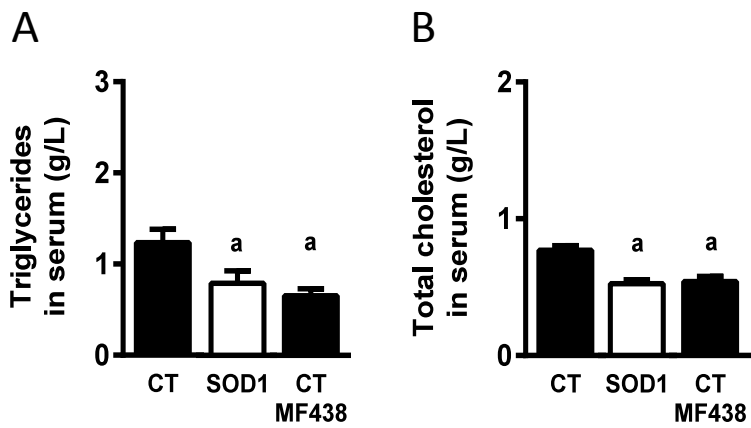


Figure 4. Lipidemia in SOD1 mice.

Triglyceride **(A)** and cholesterol levels **(B)** in symptomatic SOD1 mice and upon MF-438 treatment (n= 8; a, p<0.05).



Fatting the brain: a brief of recent research

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Fatting the brain: a brief of recent research

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Abstract

Fatty acids are of paramount importance to all cells, since they provide energy, function as signaling molecules, and sustain structural integrity of cellular membranes. In the nervous system, where fatty acids are found in huge amounts, they participate in its development and maintenance throughout life. Growing evidence strongly indicates that fatty acids in their own right are also implicated in pathological conditions, including neurodegenerative diseases, mental disorders, stroke and trauma. In this review, we focus on recent studies that demonstrate the relationships between fatty acids and function and dysfunction of the nervous system. Fatty acids stimulate gene expression and neuronal activity, boost synaptogenesis and neurogenesis, and prevent neuroinflammation and apoptosis. By doing so, they promote brain development, ameliorate cognitive functions, serve as anti-depressants and anti-convulsants, bestow protection against traumatic insults and enhance repairing processes. On the other hand, unbalance between different fatty acid families or excess of some of them generate deleterious side effects, which limit the translatability of successful results in experimental settings into effective therapeutic strategies for humans. Despite these constraints, there exists realistic evidence to consider that nutritional therapies based on fatty acids can be of benefit to several currently incurable nervous system diseases.

Keywords

brain, monounsaturated fatty acid, nervous system, neurodegenerative disease, neurological disease, peripheral nerve, saturated fatty acid, polyunsaturated fatty acid

1. Introduction

Fatty acids represent a class of lipids that are crucial components of all mammalian cells. They display a variety of biological functions to maintain vital cellular processes at various levels. Thus, fatty acids provide energy, function as signaling molecules, and sustain structural integrity of cellular membranes. They are of particular importance for the nervous system for two major reasons. First, the nervous system possesses a very high concentration of fatty acids, second only to adipose tissue (Etschmaier et al., 2011). Second, these fatty acids participate actively both in the development of the nervous system during embryonic and early postnatal life, and in its maintenance throughout adulthood and natural aging (Uauy and Dangour, 2006; Rombaldi Bernardi et al., 2012). Along with these actions, currently incurable pathological conditions of the nervous system, including neurodegenerative diseases, mental disorders, stroke and trauma, involve deregulated contents of fatty acids. It is therefore believed that these changes contribute in their own right by as yet incompletely understood mechanisms to those pathological processes. In consequence, the roles of fatty acids in health and disease of the nervous system have been intensively investigated in the last few decades. In this piece of work, we focus on recent findings that demonstrate the diversity in the fatty acids associated with function and dysfunction of the nervous system, as well as in the experimental proofs that implicate them in such tasks. The detailed mechanisms of action of fatty acids at the molecular level are not treated in this article, since they are the subject of other recently published reviews (Georgiadi and Kersten, 2012; Yamashima, 2012). To assemble the present work, we have screened for original research in the field published during the last five years, by using "central nervous system", "peripheral nervous system", "saturated fatty acids", "monounsaturated fatty acids" and "polyunsaturated fatty acids" as searching keywords.

2. Some aspects of the biochemistry of fatty acids

According to the IUPAC definition, fatty acids are "aliphatic monocarboxylic acids derived from or contained in esterified form in an animal or vegetable fat, oil or wax" (IUPAC, 1997). The naturally occurring fatty acids mostly consist of an unbranched 4-28 carbon chain that is usually composed of an even number of carbon atoms. On the basis of the carbon chain length, fatty acids are classified into short- (less than 6 carbon atoms), medium- (6-12 carbon atoms), long- (14-22 carbon atoms) and very long chain fatty acids (more than 22 carbon atoms). The fatty acids in which the aliphatic chain is fully composed of single bonds between carbon atoms are named as saturated fatty acids (SFAs), whereas the fatty acids with one or more than one carbon-carbon double bond are called unsaturated fatty acids. Based on the number of double bonds, the unsaturated fatty acids are further divided into mono- (MUFAs) and polyunsaturated fatty acids (PUFAs) (**Table 1**). Long chain SFAs have relatively high melting points that make them to appear solid at room temperature. Therefore, the body possesses a mechanism to introduce double bonds in the carbon chain, which lowers the melting point and permits functioning in a physiological environment. There are four fatty acid desaturases documented in humans that selectively catalyze the introduction of a double bond in different positions of the carbon chain. Δ -9 desaturase, also known as stearoyl-CoA desaturase, is charged with synthesizing MUFAs, mainly palmitoleic acid (16:1) and oleic acid (18:1), by introducing a double bond between carbon atoms 9 and 10 from the carboxylic acid end (**Figure 1**) (Enoch et al., 1976). Δ -4, Δ -5 and Δ -6 desaturases introduce a double bond at carbon positions 4, 5 and 6, respectively, and work cooperatively with elongases, which are responsible for the extension of the aliphatic chain. The combined actions of these desaturases and elongases are implicated in the synthesis of PUFAs (Nakamura and Nara, 2004).

According to the position of the first double bond from the methyl end of the fatty acid chain, the most important PUFAs for humans can be divided into two families: n-6 and n-3 PUFAs. LA (18:2 n-6) is the parent fatty acid of n-6 PUFAs, which produces principally AA (20:4 n-6), whereas ALA (18:3 n-3) is the parent fatty acid of n-3 PUFAs, which gives rise mainly to EPA (20:5 n-3) and subsequently DHA (22:6 n-3) (**Figure 1**). Both LA (18:2 n-6) and ALA (18:3 n-3) cannot be synthesized indigenously by the human body, so that they must be supplied with food, and such fatty acids are termed as essential fatty acids (Ruzickova et al., 2004; Lands, 2012). In spite of the fact that the body is able to metabolize these essential fatty acids, the efficiency of conversion is low. Hence, the availability not only of essential precursors but also of some of their metabolites, such as EPA (20:5 n-3) and DHA (22:6 n-3), greatly depends on dietary support (Brenna et al., 2009). Alternatively, PUFAs can also be obtained by enzymatic processing of membrane phospholipids by phospholipases (Lee et al., 2011). Whatever pathway is involved, several PUFAs can be metabolized by cyclo-oxygenases, lipo-oxygenases and cytochrome P450 mono-oxygenases to produce other compounds with important biological functions. AA (20:4 n-6) and, to a lesser extent, EPA (20:5 n-3) are transformed into potent pro-inflammatory eicosanoids. Additionally, EPA (20:5 n-3) and DHA (22:6 n-3) generate opposing anti-inflammatory docosanoids, including resolvins and protectins such as neuroprotectin-D1 (NPD1) (Bazan, 2009) (**Figure 1**).

3. Evidence of the importance of fatty acids for health and disease of the nervous system

3.1. Fatty acids and brain development

Mother's own resources, via placenta and milk, provide most of the n-3 PUFAs necessary for brain development during fetal and early postnatal life. Due to this high demand of the developing nervous system in the progeny, maternal brain levels of DHA (22:6 n-3) exhaust during pregnancy and lactation period (Chen and Su, 2012). Thus, enhanced provision or adequate supply of n-3 PUFAs at these stages can yield positive effects on offspring brain development. For instance, increased expression of neuron specific enolase, glial fibrillary acidic protein and myelin basic protein was observed in pups from mice fed on n-3 PUFA enriched diet, administered from two months prior to mouse conception to end of lactation period (Tian et al., 2011). Similarly, the postnatal supplementation of ALA (18:3 n-3), the parent precursor of n-3 PUFAs, enhanced cell proliferation and early neuronal differentiation, whilst its deprivation resulted in increased proportion of apoptosis in the dentate gyrus of unweaned pups. This ameliorating effect was offset by maternal ALA (18:3 n-3) deficiency during gestation period, suggesting that ALA (18:3 n-3) is not only required at postnatal stages but is also essential for fetal brain development (Niculescu et al., 2011). Importantly, such diets given at perinatal stages may have long lasting consequences in the adulthood. Thus, the abundance of n-3 PUFAs in the diet of pregnant females revealed essential for the development of the glutamatergic system and normal behavior performance in the adult offspring (Moreira et al., 2010a). Also, motor coordination was ameliorated in adulthood when rats were fed on DHA (22:6 n-3) and EPA (20:5 n-3) supplementation starting from gestation stage to postnatal age of 90 days (Coluccia et al., 2009). Finally, n-3 PUFA enriched diets also improved reference and working memory in offspring rats when supplied to mother at gestation stage (Chung et al., 2008).

Frequently, the impact of dietary fatty acids depends on a balance between different types of fatty acids. In a study to assess the effects of quality and quantity of several high fat diets, mice were nourished with various concentrations and types of fats mingled with normal chow. It was noticed that these diets not only modified the lipid profile in brain but also altered spatial memory and learning ability of the pups in a different manner (Yu et al., 2010). In another

study, when mice were fed on diets supplemented with either SFAs or MUFAs, MUFAs promoted insulin sensitivity and cortical activity while SFAs did not (Sartorius et al., 2012). Lastly, it is noteworthy that the intake of sufficient amount of MUFAs prevented the age related deletion of mitochondrial DNA in the brain of aged animals (Ochoa et al., 2011).

3.2. Fatty acids and neurodegenerative disorders

The altered amounts of different classes of fatty acids in the nervous system may contribute positively or negatively to any given neuropathological process (**Table 2**). Using APP-C99-transfected COS-7 cells, a cellular model of Alzheimer's disease-like degeneration, a study was carried out to investigate the class of fatty acids that was thought to influence the production of A β peptide, which is a major neuropathological hallmark of disease. It was shown that palmitic acid (16:0), stearic acid (18:0), upstream n-3 PUFAs and AA (20:4 n-6) triggered higher secretion of A β peptides compared to long chain downstream n-3 PUFAs and MUFAs (Amtul et al., 2011a). These findings were corroborated *in vivo* by using a transgenic mouse model of early-onset Alzheimer's disease that expresses the double-mutant form of human APP, which is the precursor protein responsible for the synthesis of A β peptides. Decreased levels of A β peptide and less accumulation in the form of amyloid plaques were observed in the brain of mice nourished with a diet enriched in n-3 PUFAs, mainly DHA (22:6 n-3) (Amtul et al., 2011a). Not only extraneously supplied but endogenously synthesized n-3 PUFAs can suppress the synthesis of A β peptide and the formation of amyloid plaques. Lebbadi and co-workers (Lebbadi et al., 2011) crossed 3xTg-AD mice, a model of Alzheimer's disease, with transgenic Fat-1 mice expressing a PUFA desaturase from *Caenorhabditis elegans* not present in mammals, which endogenously produces n-3 PUFAs from n-6 PUFAs. It was observed that the double transgenic 3xTg-AD/Fat-1 mice had increased brain levels of DHA (22:6 n-3) and lower levels of A β peptide. Similarly, MUFAs, mainly oleic acid (18:1 n-9), were also shown to inhibit the production of A β peptide and amyloid plaques both *in vitro* and *in vivo* (Amtul et al., 2011b). In contrast, n-6 PUFAs, such as AA (20:4 n-6), aggravated Alzheimer's disease neuropathology, by increasing the synthesis of A β peptide (Amtul et al., 2012).

The results obtained in experimental models of Alzheimer's disease have been confirmed, at a certain extent, by studies performed on human brain. Thus, decreased levels of PUFAs and MUFAs, particularly DHA (22:6 n-3) and oleic acid (18:1 n-9), respectively, were observed in the brain of Alzheimer's disease patients (Martín et al., 2010). However, other studies reported that, although the abundance of DHA (22:6 n-3) varied highly among patients, the mean quantity of this PUFA did not differ significantly when compared to healthy brains (Fraser et al., 2010). This study also showed that levels of stearic acid (18:0) were reduced remarkably in frontal and temporal cortex, while those of oleic acid (18:1 n-9) were increased in both parts; also, levels of palmitic acid (16:0) appeared increased in the parietal cortex (Fraser et al., 2010). These *a priori* puzzling abnormalities in MUFAs could be a result of alterations in the expression of MUFA synthesizing genes. Thus, levels of MUFAs in hippocampus, frontal cortex and temporal cortex were elevated in Alzheimer's disease patients, as was the expression of the stearoyl-CoA desaturase isomers SCD1, SCD5a and SCD5b. In addition, the ratio of MUFAs to SFAs, an index of desaturase activity, was reported to be negatively correlated with cognitive performance (Astarita et al., 2011).

Less is known about the changes of fatty acids in other neurodegenerative conditions. Fabelo and co-workers (Fabelo et al., 2011) reported that lipid rafts from brain cortices of patients with Parkinson disease displayed significantly decreased levels of n-3 and n-6 PUFAs, particularly DHA (22:6 n-3) and AA (20:4 n-6), respectively, while SFAs, mainly palmitic acid (16:0) and stearic acid (18:0), were noted augmented, as compared to control subjects. In

another study, the effects of diets rich in n-3 or n-6 PUFAs were assessed on cuprizone-induced experimental demyelination, an animal model for multiple sclerosis. It was observed that n-3 PUFAs from various sources affected the pathological phenotype differently; for example, a diet containing n-3 PUFAs from salmon ameliorated the behavioral deficits induced by cuprizone, whereas a diet containing n-3 PUFAs from cod affected similarly as n-6 PUFA enriched or control diet did, suggesting that not only PUFAs but their origin is also considerable when prescribing a diet based remedy (Torkildsen et al., 2009). Contrasting these findings, other studies did not corroborate the protective effects of n-3 PUFAs against multiple sclerosis and concluded that neither n-3 nor n-6 PUFAs had any effect on disease progression or remedial influence (Wergeland et al., 2012). Moreover, dietary administration of EPA (20:5 n-3) even accelerated disease progression in mice expressing a mutated form of SOD1, which is a model of neuromuscular degeneration as caused by amyotrophic lateral sclerosis (Yip et al., 2013).

3.3. Fatty acids and traumatic injury to the nervous system

Several recent studies have provided evidence that n-3 PUFAs can exert protection against neuronal injury triggered by hypoxia or ischemia. In neonates, these fatty acids protected neurons following hypoxia/ischemia injury, by modulating the microglial inflammatory response through inhibition of the NF- κ B dependent pathway (Zhang et al., 2010). However, it is important to mention that consistent increased intake of n-3 PUFAs can also affect adversely in some cases. In this respect, a diet rich in EPA (20:5 n-3) and DHA (22:6 n-3) enhanced the risk for intracerebral hemorrhagic stroke in rats, and caused oxidative damage to the brain, probably due to the fact that a high PUFA content increased the danger of lipid peroxidation. Alternatively, n-3 PUFA intake was reported to affect blood viscosity, vasoconstriction, platelet aggregation and blood clotting ultimately leading to hemorrhaging (Park et al., 2009).

There is also evidence that certain fatty acids have the potential to improve the recovery of the injured spinal cord. Hirakawa and co-workers (Hirakawa et al., 2010) reported that trans-2-decenoic acid ethyl ester, a medium-chain fatty acid derivative, increased the expression of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in cultured cortical neurons and at the site of injury in a rat spinal cord injury model. Indeed, the administration of trans-2-decenoic acid ethyl ester ameliorated functional recovery and reduced lesion size in response to injury, by increasing the expression of ERK1/2, brain-derived neurotrophic factor (BDNF), and anti-apoptotic Bcl-2. Similarly, DHA (22:6 n-3) pre-treatment in an acute spinal cord injury model diminished the extent of functional deficits as compared to that observed in the control group, and this protective effect was associated with increased survival of precursor cells, sparing of white matter and axonal preservation (Figuroa et al., 2012; Lim et al., 2013b). In the same way, mice carrying the Fat-1 transgene for boosting endogenous synthesis of n-3 PUFAs showed better outcome after spinal cord injury (Lim et al., 2013a). Finally, in relation to diabetes, it was shown that the augmentation of epoxy-fatty acid resources, as obtained by inhibiting soluble epoxide hydrolase, resulted in a dose dependent anti-allodynic effect on neuropathic pain due to glucose toxicity (Inceoglu et al., 2012).

3.4. Fatty acids and neurological disorders

Particular changes in brain fatty acid composition appear to be intimately connected to a series of neurological diseases, as recently reported by several studies. Thus, Conklin and co-workers (Conklin et al., 2010) observed a reduction in the quantity of both saturated and unsaturated fatty acids of various types, including n-3 and n-6 PUFAs, in the cingulate cortex of depressive patients. Similar alternations in n-3 PUFAs, including EPA (20:5 n-3) and DHA (22:6 n-3),

were also shown by others (Lin et al., 2010). In another study, it was noticed that the altered concentrations of MUFAs and PUFAs were region-specific. In fact, no changes in n-3 and n-6 PUFAs were found in hippocampus and orbitofrontal cortex of patients with depression but concentrations of MUFAs, such as oleic acid (18:1 n-9), and SFAs, such as palmitic acid (16:0), appeared augmented (Hamazaki et al., 2012). A partial confirmation of these findings emerged from another study showing lowered expression of genes involved in PUFA and MUFA synthesis in the frontal cortex of depressed patients (McNamara and Liu, 2011). It is also noteworthy that lifelong n-3 PUFA deficiency perturbed normal endocannabinoid function in prelimbic prefrontal cortex and accumbens, and this effect was related to impaired emotional behavior (Lafourcade et al., 2011). Although less investigated, several studies also detected changes in fatty acids in patients with schizophrenia. A decrease in docosatetraenoic acid (22:4 n-6) was observed in the amygdala of these patients but others PUFAs, including DHA (22:6 n-3) and AA (20:4 n-6), remained unchanged (Hamazaki et al., 2010; 2012). Interestingly, the decrease in total membrane PUFAs found in erythrocytes of young patients with schizophrenia correlated with the degree of demyelination in the brain white matter (Peters et al., 2009).

Lastly, several lines of evidence support the anticonvulsant effects of certain fatty acids in animal models of epileptogenesis, and the administration of PUFA enriched diets has been envisaged to treat epileptogenic convulsions. Using the pentylenetetrazol-induced epilepsy rat model, Porta and co-workers (Porta et al., 2009) showed that a PUFA containing diet increased the threshold level for pentylenetetrazol to induce convulsions. A contemporary study confirmed that rats nourished with n-3 PUFAs exhibited greater resistance to pentylenetetrazol-induced seizures (Taha et al., 2009). In addition, injection of DHA (22:6 n-3) or its derivative NPD1 into the third ventricle in the kindling model of epilepsy limited the progression in the hippocampus of the electrically induced neuronal hyperexcitability characteristic of seizures (Musto et al., 2011). In contrast, other studies did not corroborate these findings, since DHA (22:6 n-3) or EPA (20:5 n-3) showed neither anticonvulsant activity nor protection against pentylenetetrazol-induced seizures (Willis et al., 2009).

4. Cellular roles of fatty acids in the nervous system

4.1. Actions of fatty acids in the hippocampus

Many recent studies have investigated the implication of fatty acids, at cellular and molecular level, in the formation, storage and processing of memory in the hippocampus (**Figure 2**). In general, n-3 PUFAs were shown to foster neuronal activity and hence counteract memory deficits. It is well known that increased c-Fos expression is an indicator of neuronal activity in response to extracellular signals like growth factors, and it is initiated when neurons fire action potentials. Commonly, the activity of c-Fos decreases as the age extends and spatial memory goes off. Provision of n-3 PUFAs restored c-Fos expression in the hippocampus, and enhanced neuronal activity ultimately leading to the amelioration of memory deficits in aged mice (Labrousse et al., 2012). Dietary DHA (22:6 n-3) also enhanced the expression of F-ATPase involved in mitochondrial ATP synthesis in the CA1 region of the hippocampus, whereas its deficiency led to decreased GLUT1 expression and defective glucose transport in the cerebral cortex (Harbeby et al., 2012). The stimulatory action of n-3 PUFAs on gene expression also affected neurotransmission. Thus, expression of V-GLUT1 and V-GLUT2, which are implicated in glutamatergic neurotransmission, appeared increased in response to ALA (18:3 n-3) exposure (Blondeau et al., 2009). Similarly, DHA (22:6 n-3) provision to rats with traumatic brain injury enhanced learning ability, by modulating the expression levels of synapsin-1, CREB and CaMKII in the hippocampus of treated animals (Wu et al., 2008; 2011). DHA (22:6 n-3) also ameliorated spatial memory in rats by increasing the expression of subtypes of

endocannabinoid/endovanilloid receptors (Pan et al., 2011). Last, n-3 PUFAs augmented the expression of a series of transcription factors involved in learning and memory processes, including retinoic acid receptor, retinoid X receptor and peroxisome proliferator-activated receptor (Dyall et al., 2010).

In a mouse model of systemic lupus erythematosus and Sjögren's syndrome, which is characterized by behavioral abnormalities, reduced aged hippocampal neurogenesis and loss of long-term potentiation (LTP), the dietary supplementation with n-3 PUFAs corrected LTP at synapses in the medial perforant pathway/dentate gyrus and enhanced the amount of adult-born neurons in the hippocampus (Crupi et al., 2012). Similarly, DPA (22:5 n-3) also ameliorated hippocampal function by attenuating the reduction in LTP in aged brain (Kelly et al., 2011). Finally, *in vitro* studies showed that treatment of differentiated PC12 cells with EPA (20:5 n-3) resulted in activation of the neuroprotective PI3-kinase/Akt signaling pathway, a mechanism that might account for the increase in LTP observed *in vivo* following EPA (20:5 n-3) treatment (Wu et al., 2008; Kawashima et al., 2010).

In Alzheimer's disease, A β peptide induces neuronal apoptosis through degradation of the adaptor protein insulin receptor substrate-1 in a c-Jun N-terminal kinase (JNK) dependent manner. A n-3 PUFA enriched diet prevented the phosphorylation of JNK, and ultimately protected neurons from death *in vitro* and improved cognitive deficit *in vivo* (Ma et al., 2009). Also, lower levels of phosphorylated tau protein and improved brain function were observed by crossing 3xTg-AD mice with Fat-1 expressing mice to enhance the endogenous production of n-3 PUFAs (Lebbadi et al., 2011). Nevertheless, it is noteworthy that 12/15-lipo-oxygenase adversely affected Alzheimer's disease pathology by synthesizing pro-inflammatory and pro-oxidant hydroperoxyacids resulting from the oxidation of PUFAs, so that genetic ablation of this enzyme ameliorated cognitive function (Yang et al., 2010).

Neuroinflammation is one of the distinctive features of the aged or diseased brain, as demonstrated by the activation of glial cells and the increase in the expression of a variety of pro-inflammatory factors. In this respect, it was reported that n-3 PUFA provision restored spatial memory loss in aged animals by suppressing pro-inflammatory IL-1 β and reverting to normal the morphology of microglia and astrocytes in the hippocampus (Labrousse et al., 2012; Park et al., 2012). n-3 PUFAs also yielded protecting effects to neurons by blocking microglia activation in a transgenic mouse model of systemic lupus erythematosus and Sjögren's syndrome (Crupi et al., 2012). In the same way, DPA (22:5 n-3) inactivated microglia attenuating neuroinflammation and counteracting spatial learning deficit in aged brain (Kelly et al., 2011). Contrary to the protective effects of PUFAs, SFAs stimulated the secretion of pro-inflammatory cytokines and induced apoptosis in astrocytes. Particularly, palmitic acid (16:0), lauric acid (12:0) and stearic acid (18:0) triggered the secretion of TNF- α and IL-6 by engaging toll-like receptor-4. Moreover, palmitic acid (16:0) also activated caspase-3 and modified the Bax/Bcl-2 ratio in these glial cells for apoptotic demise. Interestingly, these pro-inflammatory actions of SFAs could be reverted by n-3 PUFAs like DHA (22:6 n-3) (Gupta et al., 2012; Wang et al., 2012).

Another way by which n-3 PUFAs can afford neuroprotection is by preventing apoptosis. The mouse model of infantile neuronal ceroid lipofuscinosis, a neurodegenerative disease caused by palmitoyl-protein thioesterase-1 (PPT1) deficiency, manifests enhanced endoplasmic reticulum- and oxidative stress that lead to apoptotic cell demise. In PPT1-deficient cells from such mice, intervention of n-3 PUFAs attenuated stress and repressed apoptotic death casting a protection to neuronal cells (Kim et al., 2010; Wu et al., 2011). Similarly, differentiated PC12 cells treated with EPA (20:5 n-3) showed lower rates of apoptosis and suppressed activity of

the apoptotic effector caspase-3 (Boudrault et al., 2009; Kawashima et al., 2010). Conjugated linoleic acid (18:2 n-6) also protected neurons from mitochondrial dysfunction and demise. Treatment of cortical neurons with this fatty acid following excitotoxic glutamate exposure resulted in decreased glutamate-induced loss of mitochondrial function, increased Bcl-2 expression and prolonged neuronal survival (Hunt et al., 2010). In the same manner, administration of fish oil, that is a rich source of n-3 PUFAs, protected hippocampal neurons from diabetic insult by precluding the expression of apoptosis inducing genes in both CA1 region and cultured cells, and by increasing the expression of anti-apoptotic genes, such as Bcl-2 and Bcl-xL (Zhang and Bazan, 2010; Zhao et al., 2012). Together with caspase-3, ceramides, resulting from the hydrolysis of sphingomyelin by sphingomyelinase, are well-known apoptosis inducing factors. Treatment with DPA (22:5 n-3) inactivated sphingomyelinase and caspase-3 in the hippocampus of elderly rats (Kelly et al., 2011). On the other hand, n-3 PUFA withdrawal modulated the phosphorylation of glycogen-synthase kinase-3 β and ERK1/2, predisposing more hippocampal neurons to damage in an *in vitro* oxygen and glucose deprivation model of ischemia (Moreira et al., 2010b). Along with this, a decrease in the release of PUFAs from cell membranes in the rat hippocampus, as a result of reduced phospholipase-A2 activity, caused alterations in membrane fluidity that could account for loss of spatial memory and cognitive impairment in Alzheimer's disease (Schaeffer et al., 2011). However, the protective effects of n-3 PUFAs under certain conditions seemed to be limited to some of the members of this class of fatty acids. Thus, only DHA (22:6 n-3) offset the expression of AMPA receptors in the membrane of hippocampal neurons and attenuated neurotoxicity leading to improved cognitive function. Other members of the n-3 PUFA family, especially EPA (20:5 n-3), lacked such a protective effect against AMPA-mediated toxicity (Ménard et al., 2009).

Synaptogenesis is one of the mechanisms by which memory processes take place. Hence, the loss of synapses is characteristic of neurodegenerative conditions and aging. For instance, cortical or hippocampal neurons incubated with the disease causing prion-derived peptide PrP82-146, and pre-treated with DHA (22:6 n-3) or EPA (20:5 n-3), showed less loss of synaptophysin-1 and reduced accumulation of prion peptide (Bate et al., 2010). ALA (18:3 n-3) also stimulated the expression of genes involved in synaptic functions, like VAMP-2, SNAP-25 and synaptophysin-1, that led to improved stability and physiology of synapses (Blondeau et al., 2009). Similarly, the chronic supplementation of n-3 PUFAs yielded antidepressant effects by increasing the expression of synaptophysin-1 in the hippocampus (Venna et al., 2009). However, another study performed on SH-SY5Y cells reported that DHA (22:6 n-3) did not affect the neurotransmission machinery, as evaluated by the expression of synaptotagmin-1, syntaxin-1A, and synaptobrevin-1, although the release of noradrenaline by these cells was enhanced (Mathieu et al., 2010).

Hippocampal neurogenesis also contributes to memory processing. The mouse model of systemic lupus erythematosus and Sjögren's syndrome typically exhibits age-dependent reduced hippocampal neurogenesis. Supplementation of diet with n-3 PUFAs to these mice enhanced the density of BrdU- and doublecortin positive cells in the hippocampus, suggesting an ongoing neurogenesis (Crupi et al., 2012). Similar neurogenesis enhancement was also reported in response to ALA (18:3 n-3) treatment (Blondeau et al., 2009). In addition, AA (20:4 n-6) even increased neurogenesis at postnatal stages when administered at gestation period (Maekawa et al., 2009). The importance of the stimulatory role of PUFAs for neurogenesis is also illustrated by studies reporting increased expression of fatty acid binding proteins (FABPs) in the ischemic hippocampus. FABPs are carriers of PUFAs in the cytoplasm, and their expression declines with age in association with reduced synaptic activity and other cellular functions. CA1 and dentate gyrus regions in the hippocampus showed

augmented levels of FABP-5 and FABP-7 after ischemia, suggesting elevated transportation of PUFAs in these regions to restore cellular neurophysiology (Liu et al., 2010; Ma et al., 2010). More importantly, at post-ischemic stages, the subgranular zone in the dentate gyrus of the hippocampus, a niche of adult neurogenesis, displayed a concomitant increase in the neuronal expression of FABPs and the fatty acid receptor GPR40, representing compensatory processes of newborn cells (Boneva et al., 2011a; Boneva et al., 2011b; Yamashima, 2012). Finally, it is noteworthy that many of the beneficial actions of PUFAs on hippocampal function were associated with an increase in the production of BDNF, which is a member of the neurotrophin family of growth factors involved in supporting growth, differentiation and survival of neurons (Wu et al., 2008; 2011; Blondeau et al., 2009; Venna et al., 2009; Avraham et al., 2011; Vines et al., 2012).

4.2. Actions of fatty acids in the hypothalamus

The central regulation of energy balance involves a number of neuronal circuits in the hypothalamus that either exert anorexic actions or stimulate food intake. In this respect, it was recently shown that certain fatty acids could influence the control of energy homeostasis by the hypothalamus. In general, dietary supplementation with fish oil, rich in n-3 PUFAs, normalized several hypothalamic neurochemical systems in food restricted animals (Avraham et al., 2011). However, supplementation of diet with SFAs induced endoplasmic reticulum stress and expression of cytokines via toll-like receptor-4 signaling in the hypothalamus, and this effect resulted in resistance to anorexigenic signals (Milanski et al., 2009). At the cellular level, treating hypothalamic mHy-poE-44 cells with palmitic acid (16:0) increased the expression of the orexigenic neuropeptide-Y, suggesting that this fatty acid could enhance food intake (Fick et al., 2011). Moreover, palmitic acid (16:0) faded insulin signaling and enhanced endoplasmic reticulum stress and caspase-3 cleavage in the same cell line, which resulted in apoptosis in a JNK-dependent manner (Mayer and Belsham, 2010). In another study, exposure to palmitic acid (16:0) displayed no effects on insulin resistance and inflammatory process activation but corroborated the stimulation of endoplasmic reticulum stress and apoptosis, along with the activation of mitogen-activated protein kinase (Choi et al., 2010).

4.3. Actions of fatty acids in the nigrostriatal pathway

Growing evidence supports a link between the dietary intake of n-3 PUFAs and the function (or dysfunction) of the nigrostriatal pathway involved in the control of movement (**Figure 3**). This relationship was particularly investigated in a number of animal models of Parkinson disease, which is a neurodegenerative condition primarily characterized by the loss of dopaminergic neurons connecting the substantia nigra to the striatum. In several recent studies, n-3 PUFAs were shown to be beneficial by reverting disease phenotype. In the MPTP model of Parkinson disease, pre-treatment of mice with n-3 PUFAs bestowed protection by increasing the expression of BDNF and involving its TrkB receptor (Bousquet et al., 2009; Balanzá-Martínez et al., 2011). In other studies, it was found that exposure to the n-3 PUFA ethyl-eicosapentaenoate derivative lowered the expression of Bax and caspase-3, and enhanced cortical dopamine levels (Bousquet et al., 2008; Meng et al., 2010). Furthermore, n-3 PUFAs also yielded protective influence indirectly, by attenuating inflammation-causing factors. These fatty acids targeted the NF κ B signaling pathway in microglia to suppress their over-activated response and hence protect dopaminergic neurons (Boudrault et al., 2009; Zhang et al., 2010; Ji et al., 2012; Zhou et al., 2012).

Other findings, however, did not support the beneficial effects of n-3 PUFAs on Parkinson disease. It was reported that treatment with ethyl-eicosapentaenoate, although minimized pro-

inflammatory cytokines and yielded positive effects on procedural memory deficit, it was unable to preclude the loss of nigrostriatal dopamine in MPTP mice (Shchepinov et al., 2011; Luchtman et al., 2012). Similarly, the parkinsonian neurotoxin 6-hydroxydopamine caused lesions in the medial forebrain bundle of rats and motor deficits that remained unaffected by fish oil derived n-3 PUFAs (Delattre et al., 2010). A chronic intervention of a DHA (22:6 n-3) containing diet did not modify neither the number of cortical glial cells nor the expression of α -synuclein, which is typically involved in disease pathogenesis (Muntané et al., 2010). The use of different animal models of Parkinson disease and the different ways of treating these mice to counteract the pathological process may explain the observed discrepancies. In this respect, it is important to mention that some studies indicated even adverse effects of n-3 PUFAs on Parkinson disease pathogenesis. Indeed, the presence of DHA (22:6 n-3) augmented neuritic injury and astrogliosis in mice transgenic for a Parkinson disease causing mutation in human α -synuclein. In addition, DHA (22:6 n-3) triggered oligomerisation of α -synuclein, through the activation of retinoic X receptor and peroxisome proliferator-activated receptor- γ 2. Interestingly, its withdrawal from diet was found to be beneficial against the deleterious effects caused by its provision (Yakunin et al., 2012). Finally, structural and conformational modifications in α -synuclein leading to pathological aggregation were brought by DHA (22:6 n-3) (De Franceschi et al., 2009; 2011; Bousquet et al., 2011).

4.4. Actions of fatty acids in the peripheral nerves

A subset of peripheral sensory neurons expresses TRPA1 receptor, which is a cation channel of the transient receptor potential family involved in pain and neurogenic inflammation. TRPA1 is a target for a variety of noxious and inflammatory irritant substances. In addition, it was found that n-3 PUFAs could act as a ligand for TRPA1 to excite sensory neurons and hence regulate their responses *in vivo* (Mottet and Ahern, 2012). TRPV1 receptor, which is another member of the transient receptor potential family, is also found mainly in the nociceptive neurons of the peripheral nervous system, where they are involved in the transmission and modulation of pain. In this respect, it was shown that NPD1, which has anti-inflammatory properties, inhibited TRPV1 currents induced by capsaicin in dorsal root ganglion neurons, and modulated TRPV1/TNF- α -mediated synaptic plasticity in the spinal cord, suggesting a novel analgesic role (Park et al., 2011). The effects of fatty acids on sensory neurons go beyond receptor signaling. Both n-6 and n-3 PUFAs promoted neurite outgrowth in sensory neurons from dorsal root ganglia of embryos but also adult and aged animals (Robson et al., 2010). Enhanced levels of endogenously synthesized n-3 PUFAs also bestowed beneficial effects in various aspects. Thus, dorsal root ganglion neurons from Fat-1 expressing mice exhibited more resistance to hypoxia and mechanical injury as compared to neurons from wild-type littermates. Furthermore, Fat-1 expressing mice showed better functional recovery after sciatic nerve crush. The increased endogenous levels of n-3 PUFAs reduced the expression of the stress sensor ATF-3 in dorsal root ganglion neurons, and diminished muscle atrophy (Gladman et al., 2012). Similarly, our own studies also reported that the down-regulation of SCD1, which is in charge of the production of MUFAs such as oleic acid (18:1), triggered accelerated motor function recovery after sciatic nerve crush, providing evidence for a new role of this fatty acid desaturase in modulating the restorative potential of the neuromuscular axis (Hussain et al., 2013).

The retina possesses a high concentration of n-3 PUFAs, particularly DHA (22:6 n-3). Many studies have shown that this fatty acid not only has a structural function but also protects visual neurons from trauma and disease. Recently, it was noticed that the retinal dysfunction induced by diabetes could be recovered to some extent by supplementing DHA (22:6 n-3) extraneously. In fact, diabetes resulted in reduced levels of n-3 PUFAs, by affecting n-3 fatty acid desaturase

enzymatic activity, so that the provision of a DHA (22:6 n-3) enriched diet prevented dysfunction of rods and ameliorated vision (Yee et al., 2010). Also, n-3 PUFA derived NPD1, together with pigment epithelial-derived growth factor, promoted corneal nerve regeneration in a rat model of surgical injury (Cortina et al., 2010; 2012; Kenchegowda et al., 2013). However, other studies rather obtained contradictory results. Therefore, augmented levels of DHA (22:6 n-3) bestowed no protection against retinal degeneration in mice carrying the disease causing VPP rhodopsin mutation and expressing Fat-1 (Li et al., 2009; 2010). In the same way, it was also reported that high levels of DHA (22:6 n-3) in the retina could generate oxidative stress, instead of protection, and hence enhance the susceptibility to degeneration (Tanito et al., 2009).

5. Conclusion

The biological functions of fatty acids have been investigated intensively during these last years, due to their active involvement in the physiology of both central and peripheral nervous system. They promote brain development, ameliorate cognitive functions in normal and diseased conditions, serve as anti-depressants and anti-convulsants, bestow protection against traumatic insults, and elevate repairing processes. At the cellular level, fatty acids stimulate gene expression and neuronal activity, and boost synaptogenesis and neurogenesis while preventing from neuroinflammatory toxicity and apoptosis (**Figure 2**). Although the demand for fatty acids in a healthy body applies to any of them, it can be said that, in general, excess of SFAs and, to some extent, n-6 PUFAs brings about negative consequences, whereas MUFAs and n-3 PUFAs are endowed with rather beneficial properties. This notion has resulted from a huge amount of studies that have demonstrated the good and the bad side of fatty acids in different experimental models of trauma and disease. As a matter of fact, the diversity in modeling any given physiopathological condition, together with differences in time, dose and type of fatty acid used to counteract the insult, certainly account for a number of conflicting results concerning the nature of the observed effects. In addition, it must be taken into consideration that particular fatty acids are assumed to foster neuroprotection but engender indeed a series of collateral deleterious actions, such as increasing oxidative stress susceptibility or favoring neurodegenerative protein aggregation, which may preclude the use of these fatty acids under certain (pathological) conditions (**Figure 3**). Finally, it is also noteworthy that, frequently, studies used nutritional approaches consisting in giving a specific fatty acid or its precursor mixed with others and forming part of foods relatively more complex than desired, since they also contain other substances with potential, uncontrolled positive or negative effects. Taken together, these drawbacks limit the translatability of successful results in terms of neuroprotection obtained in animal experiments into effective therapeutic interventions in humans. Numerous epidemiological studies have put fatty acids forward as key factors contributing to neuropathology but, in some cases, discrepant concentrations of fatty acids were reported in the corresponding diseased brain regions (**Table 2**). Despite these constraints, on the basis of these epidemiological studies and supported by experimental research, there is quite realistic evidence to envisage that nutritional therapies based on fatty acids can be of benefit to several neurodegenerative and neurological diseases, such as age-related macular degeneration, cognitive decline, depression and some related behavioral disorders. More research is needed now for arriving at the final and conclusive result concerning the type of fatty acid, number of double bonds, origin, particular stage and proper concentration to achieve beneficial therapeutic potential against otherwise incurable diseases.

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Conflict of interest statement

The authors declare that the work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table 1. Most typical fatty acids

Systematic Name	Common name	Abbreviation	C:D series (1)
Saturated fatty acids (SFAs)			
Butanoic	Butyric		4:0
Hexanoic	Caproic		6:0
Octanoic	Caprylic		8:0
Decanoic	Capric		10:0
Dodecanoic	Lauric		12:0
Tetradecanoic	Myristic		14:0
Hexadecanoic	Palmitic		16:0
Octadecanoic	Stearic		18:0
Eicosanoic	Arachidic		20:0
Docosanoic	Behenic		22:0
Tetracosanoic	Lignoceric		24:0
Monounsaturated fatty acids (MUFAs)			
cis-9-Hexadecenoic	Palmitoleic		16:1
cis-9-Octadecenoic	Oleic		18:1
cis-13-Docosenoic	Erucic		22:1
cis-15-Tetracosenoic	Nervonic		24:1
Polyunsaturated fatty acids (PUFAs)			
cis-9,cis-12,cis-15-Octadecatrienoic	α -Linolenic	ALA	18:3 n-3
cis-6,cis-9,cis-12,cis-15- Octadecatetraenoic	Stearidonic		18:4 n-3
cis-8,cis-11,cis-14,cis-17- Eicosatetraenoic			20:4 n-3
cis-5, cis-8,cis-11,cis-14,cis-17- Eicosapentaenoic	Eicosapentaenoic	EPA	20:5 n-3
cis-7,cis-10,cis-13,cis-16, cis-19- Docosapentaenoic	Docosapentaenoic	DPA	22:5 n-3
cis-4,cis-7,cis-10,cis-13,cis-16,cis-19- Docosahexaenoic	Docosahexaenoic	DHA	22:6 n-3
cis-9,cis-12-Octadecadienoic	Linoleic	LA	18:2 n-6
cis-6, cis-9,cis-12-Octadecatrienoic	γ -Linolenic	GLA	18:3 n-6
cis-8,cis-11,cis-14-Eicosatrienoic	Dihomo- γ -linolenic	DHGLA	20:3 n-6
cis-5,cis-8,cis-11,cis-14-Eicosatetraenoic	Arachidonic	AA	20:4 n-6
cis-7,cis-10,cis-13,cis-16- Docosatetraenoic	Docosatetraenoic		22:4 n-6
cis-4,cis-7,cis-10,cis-13,cis-16- Docosapentaenoic	Docosapentaenoic		22:5 n-6

(1) C:D nomenclature designates the number of carbon atoms in the fatty acid (C), and the number of double bonds (D). The series n-X indicates the position of the first double bond counting from the terminal methyl carbon.

Table 2. Changes in brain fatty acid composition in pathological conditions

Fatty acid	Disease	Tendency	Reference (1)
Saturated fatty acids (SFAs)			
Myristic (14:0)	Depression	Down	Conklin et al., 2010
Palmitic (16:0)	Alzheimer	Up	Fraser et al., 2010
Palmitic (16:0)	Parkinson	Up	Fabelo et al., 2011
Palmitic (16:0)	Depression	Down	Conklin et al., 2010
Palmitic (16:0)	Depression	Up	Hamazaki et al., 2012
Stearic (18:0)	Alzheimer	Down	Fraser et al., 2010
Stearic (18:0)	Parkinson	Up	Fabelo et al., 2011
Stearic (18:0)	Depression	Down	Conklin et al., 2010
Monounsaturated fatty acids (MUFAs)			
Palmitoleic (16:1)	Alzheimer	Up	Astarita et al., 2011
Palmitoleic (16:1)	Depression	Down	Conklin et al., 2010
Oleic (18:1)	Alzheimer	Down	Martín et al., 2010
Oleic (18:1)	Alzheimer	Up	Fraser et al., 2010
Oleic (18:1)	Alzheimer	Up	Astarita et al., 2011
Oleic (18:1)	Depression	Up	Hamazaki et al., 2012
Erucic (22:1)	Alzheimer	Up	Astarita et al., 2011
Nervonic (24:1)	Alzheimer	Up	Astarita et al., 2011
Polyunsaturated fatty acids (PUFAs)			
EPA (20:5 n-3)	Depression	Down	Lin et al., 2010
DPA (22:5 n-3)	Depression	Down	Conklin et al., 2010
DHA (22:6 n-3)	Alzheimer	Down	Martín et al., 2010
DHA (22:6 n-3)	Parkinson	Down	Fabelo et al., 2011
DHA (22:6 n-3)	Depression	Down	Conklin et al., 2010
DHA (22:6 n-3)	Depression	Down	Lin et al., 2010
LA (18:2 n-6)	Depression	Down	Conklin et al., 2010
AA (20:4 n-6)	Parkinson	Down	Fabelo et al., 2011
AA (20:4 n-6)	Depression	Down	Conklin et al., 2010
Docosatetraenoic (22:4 n-6)	Depression	Down	Conklin et al., 2010
Docosatetraenoic (22:4 n-6)	Schizophrenia	Down	Hamazaki et al., 2012

(1) This table summarizes recent studies cited in the text. It is concluded that, whatever disease condition is considered, PUFA levels were systematically decreased, whereas MUFA amounts often appeared increased. In contrast, there was no clear-cut tendency in the changes of the proportions of SFAs.

Figure legends

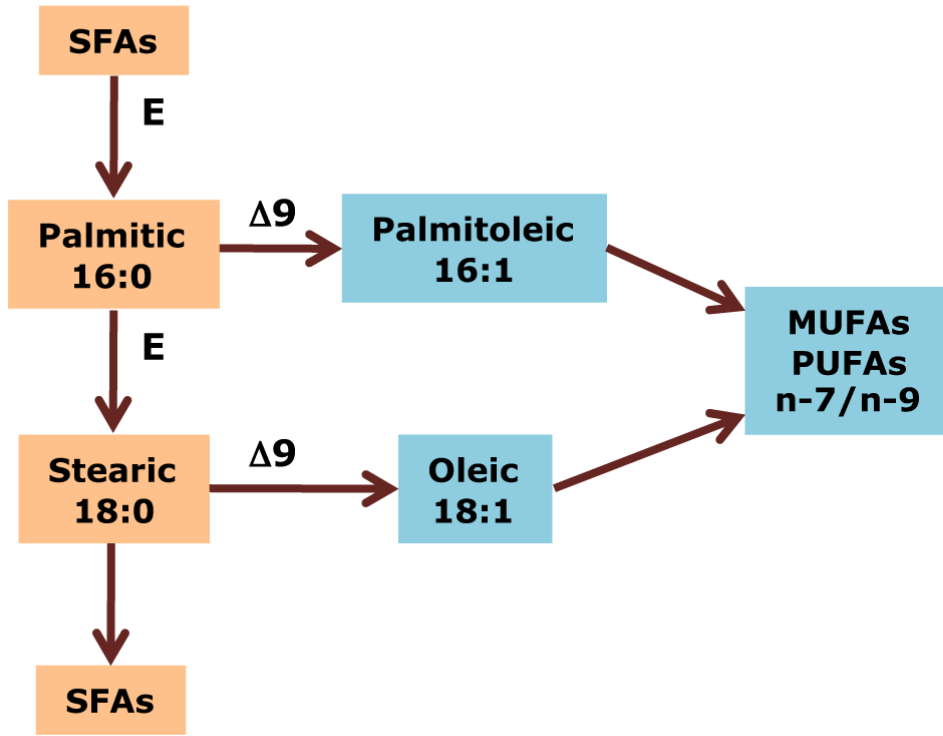
Figure 1. Biosynthesis of fatty acids. Medium- to long chain SFAs are successively transformed by the action of elongases into palmitic acid (16:0), which is then either elongated to stearic acid (18:0), and other long chain SFAs, or desaturated, together with stearic acid (18:0), by D9 desaturase to produce *de novo* MUFAs of the n-7 and n-9 series, such as palmitoleic acid (16:1) and oleic acid (18:1). In the case of PUFAs, D6 and D5 desaturases work cooperatively with elongases to introduce double bonds and extend the aliphatic chain in a successive manner, from ALA (18:3 n-3) to EPA (20:5 n-3) in the n-3 series, and from LA (18:2 n-6) to AA (20:4 n-6) in the n-6 series. Afterwards, these end products are further elongated, desaturated and submitted to peroxisomal β -oxidation (all three steps indicated by OX) to yield DHA (22:6 n-3) and docosapentaenoic acid (22:5 n-6), respectively. Finally, AA (20:4 n-6) is the precursor of potent pro-inflammatory eicosanoids. EPA (20:5 n-3) produces less potent (dashed arrow) eicosanoids and, together with DHA (22:6 n-3), gives rise to docosanoids with anti-inflammatory properties (*i.e.*, resolvins and protectins).

Figure 2. Multiple effects of fatty acids in the hippocampus. n-3 and n-6 PUFAs exert a variety of positive actions that promote formation, storage and processing of learning and memory in the hippocampus. In contrast, SFAs display rather negative actions. Green arrows indicate stimulatory effects while orange arrows represent inhibitory effects.

Figure 3. Conflicting effects of n-3 PUFAs in the nigrostriatal pathway. n-3 PUFAs are commonly endowed with a wide range of helpful effects, as illustrated by the protective benefit that these fatty acids offer to dopaminergic neurons in the nigrostriatal tract against apoptotic and pro-inflammatory cues. However, extreme caution should be exercised since these same PUFAs may not provide complete safety to halt degeneration induced by parkinsonian toxins or even trigger adverse effects, which eventually aggravates the extent of the pathological process.

Figure 1.TIF

Synthesis of MUFAs



Synthesis of n-3 PUFAs



Synthesis of n-6 PUFAs

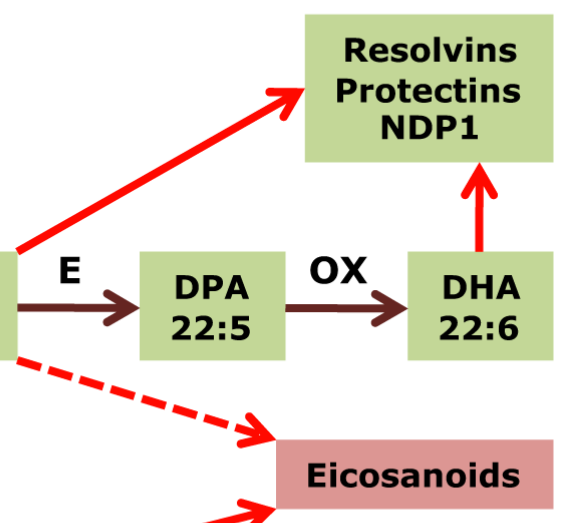
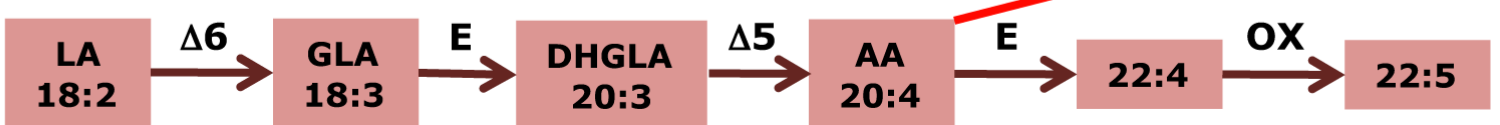


Figure 2.TIF

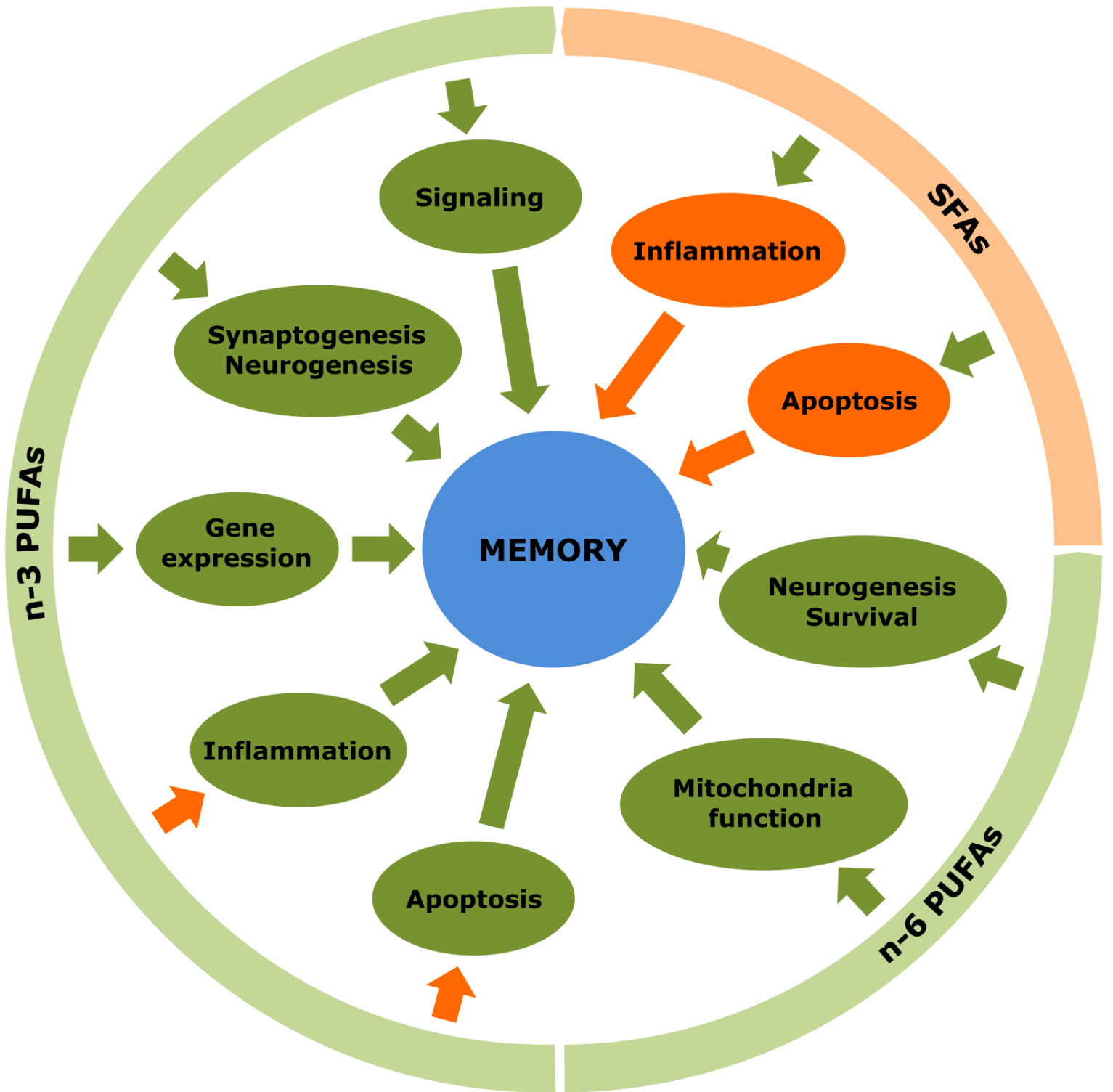
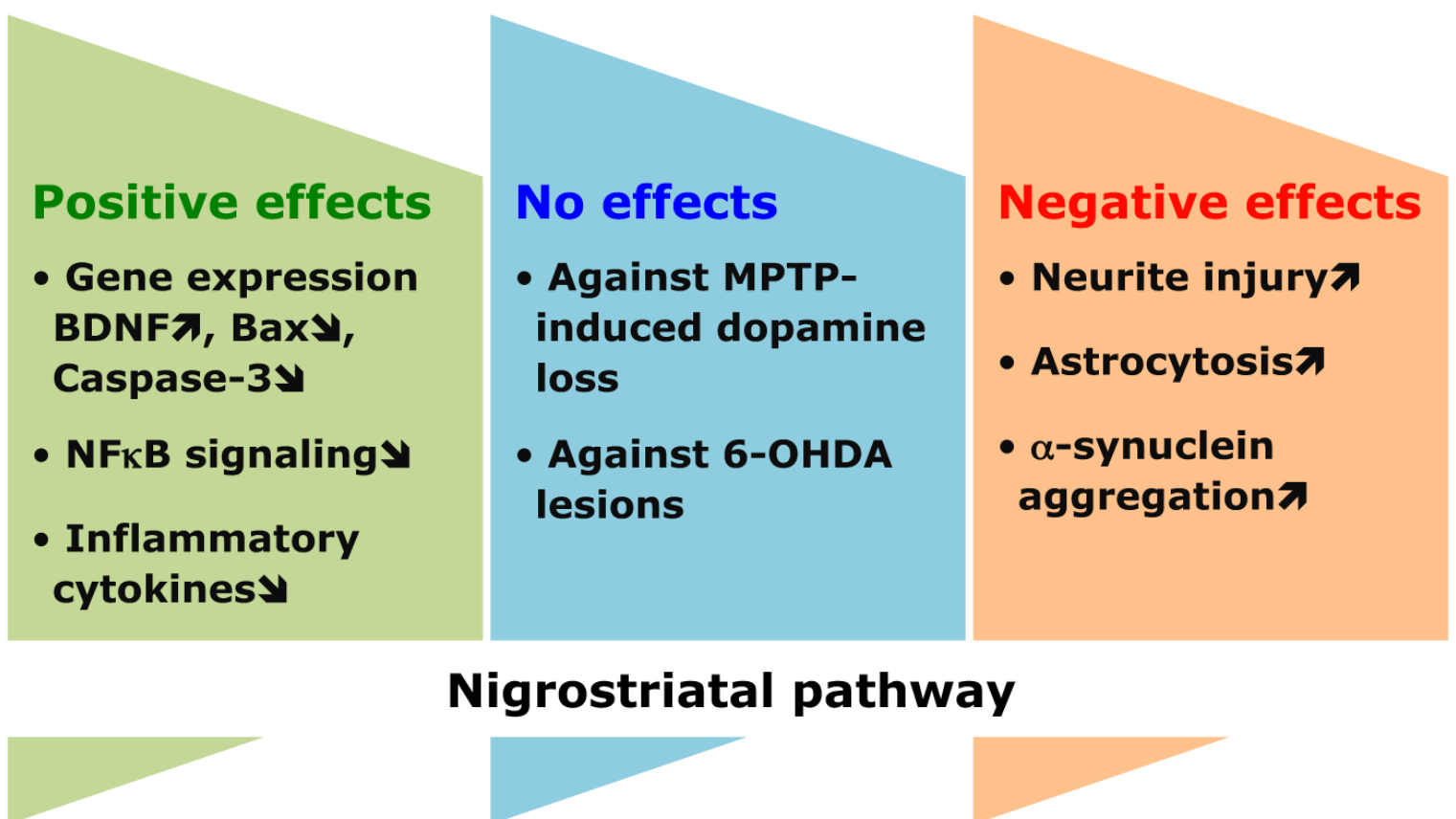


Figure 3.TIF



CONCLUSIONS AND PERSPECTIVES

Metabolic dysfunctions are noted in both ALS patients and mouse models. The origin of these alterations in circulating lipids and energy expenditure is yet to be elucidated. We have been focusing to decipher the relationship between these metabolic alterations and neurodegeneration as observed in ALS. Since muscles are atrophied in this pathology, and also are primary tissues for energy consumption therefore, muscle originated metabolic dysfunctions are concerned much in this regards. Our previous studies have shown that SOD1 mice showed an increase in energy expenditure which would probably be of muscular origin (Dupuis et al., 2004). This hypermetabolism leads to an energy deficit that may contribute to the development of the disease, because the increase in energy intake increases the survival of animals to some extent. In addition, muscle lipid catabolism in SOD1^{G86R} model seems to be increased significantly (Fergani et al., 2007). Our transcriptome study and after a review of the literature, we found that the repression of SCD1 in skeletal muscle could have an impact on the metabolic orientation of the muscle. Indeed, the striking parallelism between the metabolic phenotypes of SCD1^{-/-} mice and SOD1 mice suggests that suppression of this enzyme could play a major role in the muscle origin hypermetabolism as noted in SOD1 mice. We have already shown a significant decrease in the expression of SCD1 in mouse muscle SOD1^{G86R}. This inhibition is very early at pre-symptomatic stage. This decrease seems to be restricted to the muscles and does not affect the spinal cord. In contrast, we observed a decrease in the expression of SCD1 in the liver of mice SOD1^{G86R}, however, it can be detected at later stage, once the animals commence to display some signs of denervation at electrophysiological level but are still clinically normal.

Our previous studies have indicated that mitochondria targeted hypermetabolism in muscle is able to induce denervation and degeneration of the neuromuscular junctions (Dupuis et al., 2009). But it is noteworthy that in this mouse model, mitochondria are directly targeted and affected by UCP-1 to induce the hypermetabolism. The drawback of this model was that mitochondria were affected to yield hypermetabolism. In this regard, an early and systemic suppression of SCD1 without affecting mitochondria seems very interesting in ALS mechanistic. In addition, this reduction in SCD1 expression is not restricted to murine models, but is also found as a common hypermetabolic state in patients with sporadic and familial ALS (Bouteloup et al., 2009; Funalot et al.,

2009). Interestingly, SCD1 suppresses the synthesis of apoptosis inducing lipid metabolites such ceramide suggesting a protective effect on muscle fibers prone to die under pathological stress. All these aspects of SCD1 implication in metabolism and cell survival provoked us to study SCD1 implication in ALS scenario. Similarly, alteration in various lipid species along the course of pathology can provide a clue to assess the disease stage and can be used as biomarker for this disorder.

Talking in a more straightforward and conclusive way, our study elucidates that SCD1 plays a vital role in muscle physiology. It modulates the supply of energy and maintains muscle functions by increasing β -oxidation of fatty acids. It would be interesting to investigate whether muscle specific inhibition of SCD1 is sufficient for muscle functional recovery to the normal level in ALS conditions or systemic ablation is more beneficial.

We have also shown that the inhibition of SCD1 genetically or pharmacologically stimulates the expression of genes involved in NMJs development and functions. The expression of MuSK and cholinergic receptor units has been found elevated in SCD1 inhibited mice. These findings suggest that inhibition of SCD1 is somehow implicated in the regulation of synapse development and function. Therefore it would be of great interest to explore the mechanisms by which the effects of SCD1 presence or absence on synapses take place.

Of note, SCD1 suppression stimulates functional recovery of the muscles suffering from stress. On the basis of our results, we postulate that this beneficial effect is supported by increasing oxidative metabolism in muscles. What is happening at the nerve level will decipher the role of SCD1 inhibition on nerve regeneration following a mechanical injury as in our study. The measurement of lipid transporter proteins such as lipoprotein ApoE would explain the reason of early regeneration in SCD1 inhibited mice (Ignatius et al., 1986). Moreover, omega-3 PUFAs and ceramide synthesis affected by SCD1 gene in SCD1 knockout mice will also highlight the mechanistic causes on this improved regenerative capacity of the nerve.

Alterations in circulating and hepatic fatty acid composition resulted from the SCD1 modifications arise at a critical stage of disease onset in the ALS mouse model. This suggests that SCD1 expression functions as a sensor of very minor

alteration in the normal functioning of body tissues and in response to combat the deleterious effects of these modifications. It would be of great importance to evaluate the potential of using nutritional approaches based on the observed changes in FAs composition to palliate ALS.

SCD1 inhibition by pharmacological agent provides a protection to muscle function and, in addition, this inhibition mimics major metabolic phenotypes of mSOD1 mice. From our study it can strongly be suggested that these alterations in SCD1 expression and related altered FAs profile come forward to protect muscles against pathology. It becomes obvious that SCD1 inhibitors can be developed as a therapeutic intervention against neurodegenerative disorders such as ALS.

SUMMARY IN FRENCH

1. INTRODUCTION

La SLA est une atteinte progressive du système moteur de l'adulte, caractérisée par une dégénérescence des neurones moteurs corticaux et des motoneurones du bulbe rachidien et de la moelle épinière. Elle est associée à une atrophie musculaire sévère et progressive, responsable du décès du patient, généralement par arrêt respiratoire, entre 2 à 5 ans après l'apparition des premiers symptômes. La SLA est la maladie du motoneurone la plus fréquente chez l'adulte, et il n'existe à ce jour aucun traitement curatif de cette altération de l'axe neuromusculaire, mis à part le riluzole, une drogue anti-glutamatergique dont l'effet ne permet d'améliorer la survie du patient que de quelques mois (Kiernan et al., 2011).

Depuis quelques années, au-delà de la vision conventionnelle que l'on peut avoir de la SLA, il a été montré que des événements extérieurs au motoneurone participaient au déclenchement de sa dégénérescence. Nos travaux ont d'ailleurs été pionniers dans cet axe de recherche, car nous avons montré que les souris SOD1 mutées, un modèle de SLA, présentent une augmentation de la dépense énergétique (c'est-à-dire, un hypermétabolisme) qui provoque un déficit énergétique systémique et ce bien avant l'apparition des premiers symptômes moteurs (Dupuis et al., 2004). Ces résultats suggèrent fortement que le métabolisme, et plus particulièrement le métabolisme lipidique, joue un rôle central dans l'initiation de la pathologie.

Nos données issues de l'analyse transcriptomique dans le muscle des souris SOD1 mutées ont montré une diminution de l'expression du gène codant pour une enzyme impliquée dans le métabolisme de lipides, la stéaroyl-coenzyme-A désaturase 1 (SCD1). La SCD1 catalyse la conversion des acides gras saturés en acides gras mono-insaturés, tels que l'oléate et le palmitoléate qui sont, à leur tour, des composants majeurs des phospholipides, des esters gras et de l'ester de cholestérol (Dobrzyń and Dobrzyń, 2006). Il est intéressant de noter que l'ablation du gène *scd1* provoque une augmentation de la dépense énergétique et une diminution des réserves lipides du tissu adipeux, associées à une hausse de la consommation de lipides (Ntambi et al., 2002), des caractéristiques qui rappellent fortement le phénotype métabolique des souris SOD1 mutées (Dupuis et al., 2004; Fergani et al., 2007).

2. OBJECTIFS

Notre projet visait à déterminer le rôle de la SCD1 dans le maintien de la fonction musculaire et, éventuellement, dans la dégénérescence des motoneurones caractéristique de la SLA. Pour ce faire, premièrement nous avons étudié l'expression musculaire de la SCD1 non seulement dans le modèle animal de la SLA mais aussi dans des muscles souffrant d'une dénervation induite par une intervention chirurgicale. Deuxièmement, nous avons analysé les caractéristiques métaboliques et de la fonction motrice chez des animaux déficients en SCD1. Troisièmement, en utilisant une approche génétique mais également une approche pharmacologique, nous avons évalué l'impact de l'absence de la SCD1 sur la récupération de la fonction musculaire en réponse à une lésion du nerf sciatique. Finalement, nous avons déterminé, chez les souris SOD1 mutées, la composition en acides gras (de manière générale mais aussi particulièrement les substrats et les produits de la SCD1), dans le sang et le foie, afin de corrélérer les changements métaboliques au niveau périphérique avec la neuropathologie de la SLA.

3. CONCLUSIONS

L'analyse de l'expression musculaire de la SCD1 a montré sa diminution précoce chez des souris SOD1 mutées pré-symptomatiques. Cette expression est également diminuée dans un modèle de dénervation expérimentale induite par l'écrasement du nerf sciatique. Par contre, elle est augmentée quand la dénervation est causée par l'axotomie (section) du nerf. Ces résultats suggèrent que la diminution de l'expression de la SCD1 n'est pas liée au phénomène aigu de la perte de l'innervation après axotomie, mais plutôt au processus de restauration de la fonction musculaire, qui se met en place après l'écrasement du nerf.

Par ailleurs, l'étude des souris invalidées pour le gène *scd1*, ou de celles traitées avec un agent pharmacologique inhibant l'activité de désaturation de la SCD1, a montré que la perte de la SCD1, ou de son activité, induit une augmentation de la capacité des muscles à développer un métabolisme de type oxydatif. En parallèle, ce phénomène a été associé à une augmentation de l'expression de gènes dits "oxydatifs" (tels que PGC-1alpha and PDK4) mais également de

l'expression de gènes de la plaque motrice (tels que le récepteur à la acétylcholine et MuSK). Ces résultats suggèrent que l'absence de la SCD1 est capable de reprogrammer les caractéristiques physiologiques des muscles pour les rendre plus aptes à maintenir une jonction neuromusculaire fonctionnelle. Basés sur cette hypothèse, l'analyse du taux de restauration de la fonction musculaire après l'écrasement du nerf sciatique a démontré que les muscles déficients en SCD1 récupèrent plus rapidement face au stress.

L'étude de l'importance de la SCD1 pour la fonction musculaire nous a amené à caractériser de manière détaillée le profil des acides gras présents dans la circulation mais aussi dans le foie chez les souris SOD1 mutées. Ainsi, des analyses par chromatographie en phase gazeuse ont montré des changements dans un certain nombre d'acides gras. Le ratio acide oléique (18:1) sur acide stéarique (18:0), qui témoigne de l'activité SCD1, apparaît diminué au tout début des premiers signes électromyographiques, indiquant le commencement de la dénervation chez un animal sans attente motrice avérée. Ce même changement reste présent pendant la phase d'état de la maladie, et il est confirmé par la réduction de l'expression de la SCD1 hépatique, aussi bien au niveau de l'ARNm que de la protéine. Nous avons donc trouvé des changements qui, par exemple au niveau hépatique, indiquent un bilan métabolique général de l'animal similaire à celui obtenu par une restriction calorique.

De plus, l'inhibition de l'activité SCD1, chez des animaux normaux, par un traitement au MF-438 est suffisante pour reproduire plusieurs aspects du profil des acides gras et du phénotype métabolique caractéristiques des souris SOD1 mutées. Ceci, lié au fait que l'inhibition pharmacologique de l'activité de désaturation de la SCD1 induit également l'accélération de la récupération fonctionnelle musculaire après lésion nerveuse, laisse penser que les altérations métaboliques des souris SOD1 mutées pourraient agir contre la progression de la maladie. Nos résultats ouvrent ainsi de nouvelles voies d'exploration thérapeutique, dans le domaine des maladies neuromusculaires, et nous aident à approfondir dans la compréhension du caractère systémique de la SLA.

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Rôle de la stearoyl-coA desaturase-1 dans le maintien de l'activité musculaire: étude d'un modèle lésionnel pour la compréhension des altérations métaboliques caractéristiques de la sclérose latérale amyotrophique

Les patients SLA et les souris modèles présentent un dysfonctionnement métabolique qui coïncide avec le changement de concentration de différentes espèces lipidiques. Notre hypothèse est qu'un tel dysfonctionnement métabolique au niveau musculaire conduirait aux premiers changements observés dans la SLA. Nous avons montré que l'expression de la stéaroyl-coenzyme A désaturase 1 (SCD1), une enzyme clé de la synthèse des acides gras mono-insaturés à partir des acides gras saturés, est diminuée dans le muscle avant les premiers symptômes moteurs observés chez les souris modèles de SLA. Dans ce modèle murin, les altérations en acides gras au niveau circulant et hépatique, traduisant les changements de SCD1, apparaissent lors des premiers symptômes de la pathologie. De plus, l'inhibition pharmacologique de l'activité de SCD1 mime le phénotype métabolique des souris modèles de SLA. Notre étude a ainsi montré que la diminution de la SCD1 joue un rôle important pour l'activité neuromusculaire. Elle module les besoins énergétiques, maintient l'activité musculaire par augmentation du métabolisme oxydatif et agit sur l'expression de gènes impliqués dans le développement et le fonctionnement de la jonction neuromusculaire. De plus, l'ablation du gène SCD1 stimule la récupération fonctionnelle musculaire après lésion du nerf. L'inhibition pharmacologique de SCD1 apporte également une protection au muscle. Nous avons pu conclure de cette étude qu'une modification de l'expression de SCD1 ainsi que du profil d'acides gras peut apporter une protection au muscle pour lutter contre la pathologie. En outre, des inhibiteurs de l'activité enzymatique de la SCD1 pourraient être développés comme traitement thérapeutique dans la SLA.

ALS patients and mouse model manifest metabolic dysfunctions that coincide with the modified levels of various lipid species. We postulated that metabolic dysfunctions in muscles function as a leading preliminary change in ALS. We have noted that the expression of stearoyl-CoA desaturase 1 (SCD1), a key enzyme that synthesises monounsaturated fatty acids (MUFAs) from saturated fatty acids (SFAs), is diminished even at pre-symptomatic stage in the muscles of an ALS mouse model. In these mice, alterations in circulating and hepatic fatty acid composition, resulting from SCD1 modification, arise at a critical stage of disease onset. Of note, inhibition of SCD1 enzymatic activity by a specific pharmacological agent mimics the metabolic phenotype of the ALS mouse model. Our study also elucidates that the lack of SCD1 plays a vital role in neuromuscular function. It modulates energy supply, and maintains muscle activity by increasing oxidative metabolism and the expression of genes involved in neuromuscular junction development and function. In addition, ablation of SCD1 gene stimulates functional recovery of muscles after a nerve lesion. Pharmacological SCD1 inhibition also provides a protection to muscle function. We conclude that alteration in SCD1 expression and related altered fatty acid profile may protect muscles against pathology. Therefore, SCD1 inhibitors can be developed as a therapeutic intervention.