

“Compositions comprising amino acids for use and treatment of central nervous system injuries”

Field of the invention

5 The present description relates generally to compositions comprising amino acids. More particularly, the description relates to compositions comprising amino acids for use in the treatment of central nervous system injuries in a subject.

Background

10 The central nervous system (CNS) comprises the brain, coordinating higher-level functions, and the spinal cord serving mainly as the communication pathway between the brain and the periphery. Disabilities from central nervous system's injuries are a function of the mode, severity, and anatomical location of the insult. Regardless of the initial location of the insult to the CNS, injury is an ongoing process, with primary damage leading to a cascade of deleterious events that can
15 affect both cell body and axonal function, resulting in continued dysfunction and prolonged degeneration.

 Traumatic brain injury (TBI) and traumatic spinal cord injury (SCI) occur when an external physical insult causes damage and can range from mild to severe. Spinal cord injury is cause of disability with devastating neurological
20 outcomes and limited therapeutic opportunities. There are two major types of spinal cord injury (SCI), transection of the spinal cord and spinal cord contusion. SCI results in partial or complete loss of motor or sensory functions below the level of the injury, with devastating consequences for the patient both at the physical and psychological level. The pathological basis underling the diseases is
25 the loss of neural tissue that disconnects the neuronal impulses from the central nervous system to the muscles. Worldwide approximately 250,000-500,000 people suffer an injury to the spinal cord every year (corresponding to 30-60 people every hour). These patients require extensive medical assistance throughout their life, with an estimated lifetime cost of 1-4 million euros per
30 patient. Although SCI represent an important social and economic burden, few new therapeutic options are available. In the last 30 years, progress in medical assistance has increased the survival rate of SCI patients. Basic and translational research, however, did not succeed in substantial improving the neurological outcome.

35 **Summary of the invention**

The present description provides amino acid-based compositions particularly effective in the treatment of central nervous system injuries in a subject.

According to the present description, the above object is achieved thanks
5 to the subject matter specifically recalled in the ensuing claims, which are understood as forming an integral part of this disclosure.

An embodiment of the present description provides a composition for use in the treatment of a central nervous system injury in a subject, the composition comprising an active agent, said active agent containing the amino acids leucine,
10 isoleucine, valine, threonine, lysine and the carboxylic acids citric acid, succinic acid, malic acid.

In one or more embodiments, the active agent of the composition further contains one or more amino acids selected in the group consisting of histidine, phenylalanine, methionine, tryptophan, cysteine and tyrosine.

15 In a preferred embodiment, the central nervous system injury is selected in the group consisting of cerebral injury, spinal cord injury, peripheral nerve injury, demyelinating disease. In one or more embodiments, the spinal cord injury comprises contusive spinal cord injury.

A further embodiment of the present disclosure provides a method of
20 treating central nervous system injuries in a subject, the method comprising selecting a composition comprising an active agent, said active agent containing the amino acids leucine, isoleucine, valine, threonine, lysine, and the carboxylic acids citric acid, succinic acid, and malic acid, and administering the composition in order to treat the central nervous system injuries.

25 **Brief description of the drawings**

The invention will now be described, by way of example only, with reference to the enclosed figures, wherein:

- Figure 1 shows a quantification of mitochondrial area in differentiating NSCs *in vitro* supplemented with a composition according to embodiments of the
30 instant disclosure. Data are presented as mean $\pm$ SEM. $*=p<0.05$; $**=p<0.01$;

- Figure 2 shows a quantification of the oxidative metabolism in differentiating NSCs *in vitro* supplemented with a composition according to embodiments of the instant disclosure. The Seahorse extracellular flux analyzer allows the measurement of different bioenergetics parameters by sequential
35 injection of different inhibitors of the mitochondrial electron transport chain.

Rotenone and Antimycin-A injection allows the calculation of non-mitochondrial OCR and the baseline mitochondrial respiration. Oligomycin injection allows measuring of respiration coupled to ATP synthesis and maximal respiration is induced by the uncoupler FCCP. Data are presented as mean $\pm$ SEM. **= $p < 0.01$;

5 ***= $p < 0.001$; ****= $p < 0.0001$;

- Figure 3 shows a pattern of neuronal maturation of differentiating NSCs *in vitro* supplemented with a composition according to embodiments of the instant disclosure. The graphs in A and B show quantitative analysis of MAP2⁺ Neurons at 10 DIV in control, ALPHA5 0.1%, ALPHA5 0.5% and ALPHA5 1% conditions. Data are presented as mean $\pm$ SEM. *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$;

- Figure 4 shows the dendritic spine maturation and excitatory synaptic markers expression in differentiating NSCs *in vitro* supplemented with a composition according to embodiments of the instant disclosure. Data are presented as mean $\pm$ SEM. *= $p < 0.05$; **= $p < 0.01$; ***= $p < 0.001$; ****= $p < 0.0001$;

- Figure 5 shows the weights of the animals at the time of surgery in three different set of experiments (BCaa1-SCI, BCaa2-SCI, BCaa3-SCI). Data are shown as mean $\pm$ SEM. SCI= spinal cord injury;

20 - Figure 6 shows the result of the locomotor functional recovery analysis in control- and treated-animals after severe SCI. The graph represents the BMS score of composition-treated (line with triangles) and control (line with squares) after severe SCI. The arrow indicates the starting day of the composition administration (3 dpi). Injured mice received the supplementation in sterile water (n=22 among 3 experiments) or only sterile water (control mice; n=25 among 3 experiments). Data are shown as mean $\pm$ SEM. *= $p \leq 0.05$, **= $p \leq 0.01$, ***= $p \leq 0.001$, ****= $p \leq 0.0001$. BMS = basso mouse scale; dpi = day post injury;

- Figure 7 shows a graph representing the ankle movement score in control- (line with squares) and treated-mice (line with triangles) after severe SCI. Data are shown as mean $\pm$ SEM. **= $p \leq 0.01$, *= $p < 0.05$. BMS=basso mouse scale; dpi=day post injury;

- Figure 8 shows a graph representing the daily diuresis of control- (line with squares) and treated-mice (line with triangles) after severe SCI. Data are shown as mean $\pm$ SEM; dpi = day post injury;

35 - Figure 9 represents transversal spinal cord immunostained slices showing

- glial scar reaction and Cyst area in control- and treated-mice after severe SCI. Transversal spinal cord slices immunostained with the specific astrocytic marker GFAP and TO-PRO3TM of a control- (A) and a treated-mouse (B) illustrating the regions considered for the quantifications. Transversal spinal cord slices
- 5 immunostained with the specific astrocytic marker GFAP and TO-PRO3TM of a control- (C) and of a treated-mouse (D). The gray dashed area indicates the glial scar area. The white dashed area indicates the cyst area. White dashed area and grey dashed area (delineated with a white line) indicate the total lesion area. (E) Graph representing the percentage ratio of the cyst area on the total lesioned area
- 10 in a control and a treated-mouse. (F) Graph representing the percentage ratio of the glial scar area on the total lesion area in control- and a treated-mouse. Quantitative data are expressed as means $\pm$ SEM; * $p \leq 0.05$; ** $p \leq 0.01$. $n=3$ animals and 8 slices/animal were analysed for each group. GFAP=Glial Fibrillary Acidic Protein;
- 15 - Figure 10 shows a graph representing the percentage ratio of the glial scar area on the total slice area in control- and treated-mice after severe SCI. Quantitative data are expressed as means $\pm$ SEM; * $p \leq 0.05$. $n=3$ animals and 8 slices/animal were analysed for each group;
- Figure 11 shows a graph representing the number of NeuN-positive cells
- 20 in the spinal cord of treated and control animals in the perilesioned area. Data are shown as mean $\pm$ SEM; $n=3$ animals and 8 slices/animals were analysed for each group. ** $P < 0.01$;
- Figure 12 shows a graph representing the percentage ratio of the area covered by Neurofilament-200 on the selected region of interest (ROI) in treated
- 25 and control mouse. Quantitative data are expressed as means $\pm$ SEM; SCI= spinal cord injury; $n=3$ animals and 8 slices/animals were analysed for each group;
- Figure 13: in panel A and B spinal cord transversal slices stained with Luxol Fast Blue (LFB) of a treated and a control mouse respectively. LFB allows the identification of the myelin content in the spinal cord parenchyma, from the
- 30 demyelinated area (white area). C) The graph represents the percentage of the myelin content in the dorsal column of the spinal cord sections of treated- and control-mice calculated as myelin positive pixels in the dorsal column among the pixels of the total area of the dorsal column. Data are shown as mean $\pm$ SEM; $n=3$ animals and 8 slices/animals were analysed for each group. ns=not significant.
- 35 SCI=spinal cord injury.

Detailed description of preferred embodiments

In the following description, numerous specific details are given to provide a thorough understanding of embodiments. The embodiments can be practiced without one or more of the specific details, or with other methods, components, materials, etc. In other instances, well-known structures, materials, or operations are not shown or described in detail to avoid obscuring aspects of the embodiments.

Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment. Thus, the appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments. The headings provided herein are for convenience only and do not interpret the scope or meaning of the embodiments.

The Inventor of the instant application surprisingly found that by adding specific carboxylic acids in specific amounts to a composition comprising a combination of leucine, isoleucine, valine, threonine and lysine a high effectiveness in favoring and increasing oxidative metabolism in differentiating neuronal cells occurred.

Feature of differentiated mature neurons is the switch from glycolysis to a more oxidative metabolism (Zheng et al., 2016). In order to investigate whether the supplementation of the composition herein disclosed could enhance this metabolic switch (pro-oxidative metabolism) and promote neuronal differentiation, the effect of the composition supplementation on differentiating sub-ventricular zone (SVZ)-derived NSCs was analyzed. In addition, to further investigate whether the composition herein disclosed affects the neuronal cell differentiation and maturation in NSCs from other origin, the analysis was expanded on neurogenic Radial-Glia Like Cells present in meninges (Bifari et al., 2017c).

Meningeal NSCs and SVZ-derived NSCs were isolated as previously described (Bifari et al., 2017) and sorted for the expression of the markers PDGFR β and CD133/Prominin-1, respectively.

After culturing, meningeal-NSCs and SVZ-derived-NSCs were expanded

as neurospheres and differentiated into neurons by changing media conditions (Bifari et al., 2009). The media were supplemented with increasing concentration of the composition according to embodiments of the instant application, starting from 0.1%, to 0.5% and 1%.

5 The composition according to embodiments of the instant application has been shown to enhance the neuronal maturation *in vitro*, as disclosed in the “Result section”.

 In addition, following the very promising and surprising results obtained *in vitro*, the Inventor also tested the effect of the composition administration on a
10 preclinical animal model of complete (severe) contusive spinal cord injury (SCI).

 SCI results in partial or complete loss of motor or sensory functions below the level of the injury, with devastating consequences for the patient both at the physical and psychological level.

 The composition herein disclosed - comprising as active agent the amino
15 acids leucine, isoleucine, valine, threonine, lysine in combination with three carboxylic acids, which are substrates of tricarboxylic acid cycle, including citric acid, succinic acid, and malic acid in specific amounts - has been shown to be very effective in counteracting the neural tissue loss and favouring the neuronal regeneration after complete contusive SCI, i.e. an injury that has no spontaneous
20 recovery and mostly reflect the condition observed in humans. The compositions herein disclosed are significantly more effective than a similar amino acid composition free of such specific carboxylic acids.

 In one or more embodiments, in the composition herein disclosed the weight ratio between the total amount of citric acid, succinic acid and malic acid
25 and the total amount of the amino acids leucine, isoleucine, valine, threonine, lysine is comprised between 0.05 and 0.3, preferably between 0.1 and 0.25.

 In one or more embodiments, the active agent may further comprise one or more amino acids selected in the group consisting of histidine, phenylalanine, methionine, tryptophan, cysteine, and tyrosine.

30 In one or more embodiments, the carboxylic acids contained in the composition may consist of citric acid, succinic acid, and malic acid.

 In a further embodiment, the active agent of the composition herein disclosed may also include aspartic acid and/or ornithine L-alphaketoglutarate (OKG).

35 According to an embodiment, the composition comprises an active agent,

the active agent consisting of leucine, isoleucine, valine, threonine, lysine, histidine, phenylalanine, methionine, tryptophan, cysteine, and optionally tyrosine, as well as citric acid, succinic acid, and malic acid, said amino acids being the sole amino acids contained in the composition. Citric acid, succinic acid, and malic acid may be the sole carboxylic acids contained in the composition.

In a further embodiment, the composition may comprise the amino acids isoleucine, leucine, and valine in an amount between 35% and 65% by weight, preferably between 42% and 56% by weight, with respect to the active agent weight.

In one or more embodiments, the weight ratio between leucine and citric acid is comprised between 5 and 1, preferably between 2.50 and 3.50.

In a further embodiment, the weight or molar amount of citric acid is higher than the weight or molar amount of each of malic acid and succinic acid. Preferably, the weight or molar amount of citric acid is higher than the weight or molar overall amount of malic acid plus succinic acid. In a further embodiment, the weight ratio between citric acid and the sum of malic acid and succinic acid is comprised between 1.0 and 4.0, preferably between 1.5 and 2.5. In a preferred embodiment, the citric acid:malic acid:succinic acid weight ratio is comprised between 10:1:1 and 2:1.5:1.5, preferably between 7:1:1 and 1.5:1:1, more preferably between 5:1:1 and 3:1:1. In a preferred embodiment the citric acid:malic acid:succinic acid weight ratio is 4:1:1.

According to some embodiments of the present disclosure, the preferred isoleucine:leucine molar ratio is comprised in the range 0.2-0.7, preferably in the range 0.30-0.60 and/or the preferred valine:leucine weight ratio is comprised in the range 0.2-0.70, preferably in the range 0.30-0.65.

In a further embodiment, the threonine:leucine molar ratio is comprised in the range of 0.10-0.90, preferably in the range 0.20-0.70 and/or the lysine:leucine weight ratio is comprised in the range of 0.20-1.00, preferably in the range 0.40-0.90.

In a preferred embodiment, the ratio between the overall molar amount of citric acid, malic acid, succinic acid and the overall molar amount of methionine, phenylalanine, histidine and tryptophan is higher than 1.35.

In one or more embodiments, the weight ratio between the sum of citric acid, malic acid, succinic acid and the sum of the branched chain amino acids

leucine, isoleucine, valine is comprised between 0.1 and 0.4, preferably between 0.15 and 0.35.

In a further embodiment, the overall weight amount of the branched-chain amino acids leucine, isoleucine, valine plus threonine and lysine is higher than the
5 overall weight amount of three carboxylic acids, such as citric acid, malic acid, and succinic acid. Preferably, the weight amount of the single carboxylic acid (citric acid, succinic acid, or malic acid) is less than the weight amount of each of the single amino acids leucine, isoleucine, valine, threonine, and lysine.

In a further embodiment, the overall molar amount of lysine and threonine
10 is higher than the overall molar amount of the three carboxylic acids citric acid, succinic acid, malic acid. Preferably, the ratio between the overall molar amount of the three carboxylic acids citric acid, succinic acid, malic acid and the overall molar amount of lysine and threonine is comprised between 0.1 and 0.7, preferably between 0.15 and 0.55.

15 In one or more embodiments, the composition herein disclosed further comprises vitamins, preferably selected in the group of vitamins B, such as vitamin B₁ and/or vitamin B₆. In a further embodiment of the present disclosure, the composition may include carbohydrates, additives and/or flavoring substances.

In one or more embodiment, the compositions herein disclosed are
20 intended for use in the treatment of central nervous system injuries preferably selected in the group consisting of cerebral injury, spinal cord injury, peripheral nerve injury, demyelinating disease. The spinal cord injury may comprise contusive spinal cord injury.

When preparing the compositions according to the instant disclosure, and
25 specifically the active agent, the amino acid arginine is preferably avoided. In addition, further amino acids preferably avoided by the composition herein disclosed may be serine, proline, alanine. Such amino acids can be counterproductive or even harmful in some concentrations or stoichiometric ratios within the composition.

30 The amino acids disclosed in the instant application can be replaced by respective pharmaceutically acceptable derivatives, namely salts.

According to a further embodiment, the amino acid compositions may
comprise pharmaceutically acceptable excipients, like for example proteins, vitamins, carbohydrates, natural and artificial sweeteners and/or flavouring
35 substances. In a preferred embodiment, the pharmaceutically acceptable

excipients may be selected from whey proteins, maltodextrins, fructose, calcium caseinate, fish oil, sucralose, sucrose esters, vitamin D3, group B vitamins.

For oral use, the compositions according to the description may be in the form of tablets, capsules, granules, gel, gelable powder, or powder.

5 The composition herein disclosed may be administered in a subject in dosage/day of active agent ranging from 8.0 g to 24.0 g.

The disclosure also provides a method of treating central nervous system injuries in a subject, the method comprising selecting a composition comprising an active agent, said active agent containing the amino acids leucine, isoleucine, 10 valine, threonine, lysine, and the carboxylic acids citric acid, succinic acid, and malic acid, and administering the composition in order to treat the central nervous system injuries.

Further specifications, in terms of amounts and ratios among the various amino acids provided for by the compositions are contained in the attached 15 claims, which form an integral part of the technical teaching provided herein in relation to the invention.

EXAMPLES

Table 1 shows the amino acid-based composition named “alpha 5m (α5m)” tested *in vitro* and *in vivo*.

20

Table 1

Composition (% w/w)	α5m
L-Leucine	31,0885
L-Lysine HCl chlorhydrate	16,903
L-Isoleucine	10,3628
L-Valine	10,3628
L-Threonine	7,254
L-Cysteine	3,1089
L-Histidine	3,1089
L-Phenylalanine	2,0726
L-Methionine	1,0363
L-Tyrosine	0,6218
L-Tryptophan	2,0726
Vitamin B1 (thiamine chlorhydrate)	0,004

Vitamin B6 (pyridoxine chlorhydrate)	0,0038
Citric acid anhydrous	8,0000
Malic acid	2,0000
Acid L-aspartic	-
Succinic acid	2,0000
Ratio Leucine:Isoleucine:Valine	3:1:1

The $\alpha 5m$ composition may be prepared first by sifting all the components with a 0.8 mesh. To obtain a pre-mixture, each ingredient (in an amount <10% by weight of the total amount) is put in a polyethylene bag together with a portion of L-lysine HCl so as to obtain 10% of the weight of the total composition. The bag is then manually shaken for 5 minutes. The pre-mixture is then loaded in a mixer (Planetaria) together with the remainder of the ingredients and mixed for a period of 15 minutes at 120 rpm to obtain a homogeneous final composition.

METHODS

Cell Culture and Treatments

Cell cultures of meningeal NSCs, sub ventricular zone (SVZ)-derived NSCs, (men-Neurons and SVZ-Neurons) were supplemented with $\alpha 5m$ composition ($\alpha 5m$). $\alpha 5m$ was added to the specific culture medium (0.1%, 0.5%, 1%), then medium pH was adjusted at 7.4 and filtered before being added to the different cell cultures. $\alpha 5m$ was changed every 2-3 days and supplemented up to maturation of the men-Neurons and SVZ-Neurons at 10 days in vitro (DIV).

Cell culture immunofluorescence

First, cells were plated onto poly-D-lysine coated glass slides. After a fixation step in 4% paraformaldehyde (PFA, Sigma-Aldrich), aspecific binding sites were blocked by incubation in blocking solution (3% fetal bovine serum, 1% bovine serum albumin, 0.3% Triton X-100 in PBS 1X). Subsequently, cells were incubated in primary antibody solution (specific primary antibodies in blocking solution) for 1.5 h at room temperature, washed twice with blocking solution and incubated in the specific secondary antibody solution (specific secondary antibodies in blocking solution) for 1h. Following three washes in blocking solution, slides were incubated with the nuclear dye TO-PRO3 (Thermo Fisher Scientific) for 10 minutes and mounted on glass microscope slides for confocal microscope quantification (Zeiss LSM 710 confocal microscope). Primary

antibody: Microtubule-associated protein 2 (MAP2 rabbit, Sigma, 1:200) to label mature neurons, TOMM20 (mouse, 1:200, Abcam) to label mitochondria. The following secondary antibodies were used: donkey anti-rabbit Alexa Fluor 546 (Molecular Probes, 1:500), goat anti-rabbit Alexa Fluor 488 (Molecular Probes, 1:500).

Animals and surgical procedures

Experimental protocols were approved and conducted in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the Italian Ministry of Health, and complied with The National Animal Protection Guidelines.

Adult female C57BL/6J (7 weeks old) mice were exposed to laminectomy and the contusive injury (SCI) was performed at vertebrate thoracic level 11 (T11). Three different experiments with 18, 20 and 12 animals respectively were performed.

Before performing every SCI surgery, all animals were weighed and only mice that weigh in a range of 16 – 21 gr were considered for the experiments. Weights of animals at the time of the surgery are shown in Figure 5.

The procedure of severe SCI, due to the severity of the performed surgeries or to complication during the recovery period caused the death of 1 animal out of 50.

Animals were housed separately in clean polypropylene cages and divided into 2 groups: 1) control-fed group (CTRL, n = 22); 2) α 5m-fed group (α 5m, n = 25), i.e. the group of animals fed with the amino acid composition shown in Table 1.

Locomotor evaluation

In order to evaluate the effects of the α 5m supplementation on the motor functional recovery after a severe SCI, the Basso Mouse Scale (BMS) analysis was performed (Basso et al., 2006). The locomotor performance of each mouse was assessed at 1, 3, 5, 7 dpi during the first week after the SCI surgery and twice a week (10, 14, 17, 21, 28, 31 dpi) till the end of the experiment (31 dpi), when mice were perfused and spinal cords were dissected. Only mice that show a BMS score of “0” at 1 dpi were included in the analysis, mice with higher BMS score were excluded from the study.

Ankle movement analysis

This parameter allows to determine if the treatment applied shows a

beneficial effect avoiding the instauration of a spastic condition of the muscles related to the ankle joint movement. In this analysis, a score to the movement of both the left and right ankle joint was assigned accordantly to the quality of their movement: “0” corresponds to no movement (spastic condition) “0.5” to a not complete movement, and “1” to a normal movement.

Bladder functionality evaluation

The diuresis of the animals was analysed starting from 1 dpi till the end of the experiment. Bladders were emptied manually and the urine was collected and weighed twice a day till 5 dpi and then once a day till the end of the experiment.

Tissue histology and immunohistochemical analysis

Spinal cord fixation and processing

Animals were anesthetised with intraperitoneal injection of chloral hydrate (2,2,2-Trichloroethane-1,1-diol, 2 ml/kg) and intracardially perfused with saline solution (0.9% NaCl) followed by 4% paraformaldehyde (PFA, Sigma–Aldrich) added with 4% sucrose in PBS 1X. Spinal cords were extracted and post-fixed overnight in 4% PFA/4% sucrose. Then they were stored in 30% sucrose solution at 4°C to cryoprotect the tissues. For histochemical analysis, 1.5 cm of dissected spinal cords (0.75 cm rostral and 0.75 cm caudal from the lesion site) cord were embedded with Optimal Cutting Temperature compound (OCT) and cryosectioned (25 µm-thick transverse sections or 20 µm-thick longitudinal sections). The obtained sections were stored at -20°C and analysed with histological and immunohistochemical methods.

Luxol Fast Blue Staining (Fig. 13)

Myelin content was quantified in the tissue sections via Luxol Fast Blue (LFB) staining. First, 0.1% LFB solution was prepared solubilizing LFB (Sigma-Aldrich) in 95% ethanol (EtOH, Carlo Erba) and 1.22% glacial acetic acid (Carlo Erba). Then, sections were hydrated in EtOH solutions (100%, 95%, 70% and 50%) and stained with 0.1% LFB solution at 40°C for 40 minutes. They were then rinsed with tap water and differentiated in 0.05% Li₂CO₃ solution (Sigma-Aldrich), dehydrated in EtOH solutions (50%, 70%, 95% and 100%), cleared in xylene (Carlo Erba) and mounted with Entellan (Merck-Millipore) for light microscopy analysis of myelin content (Zeiss Axioscop2).

Spinal cord tissue analysis

Cryosections were thawed, washed with PBS 1X and incubated for 30 minutes in blocking solution (0.25% Triton X-100, 2% bovine serum albumin in

PBS 1X) in order to block aspecific binding sites. Slides were then incubated in primary antibodies solution (specific primary antibodies in blocking solution) overnight at 4°C. After rinsing 6 times for 5 minutes in blocking solution, slides were incubated in the specific secondary antibody solution (specific secondary antibodies in blocking solution) for 4 hours at room temperature. Following three washes in blocking solution and three in PBS 1X, slides were incubated with the nuclear dye TO-PRO3 (Thermo Fisher Scientific) or 4',6-Diamidino-2-Phenylindole (DAPI, Thermo Fisher Scientific, 1:2000) for 10 minutes and mounted using 1.4-Diazabicyclo (2.2.2) octane (DABCO, Sigma- Aldrich) on for microscope quantification (Zeiss LSM 710 confocal microscope). Primary antibodies used: Glial Fibrillary Acidic Protein (GFAP goat, 1:200, Abcam) to label astrocytes (Fig. 9), Neurofilament-200 (NF200 rabbit, 1:200, Sigma) to label neurofilament protein 200 KDa of the neuronal cytoskeleton (Fig. 12), NeuN (NeuN mouse, Chemicon, 1:200) to label neurons (Fig. 11). Secondary antibody used: donkey anti-rabbit Alexa Fluor 546 (Molecular Probes, 1:500), goat anti-rabbit Alexa Fluor 488 (Molecular Probes, 1:500), donkey anti-mouse Alexa Fluor 488 (Molecular Probes, 1:500), goat anti-mouse CY3 (Amersham, 1:500), donkey anti-goat Alexa Flour 488 (Life-technologies, 1:500).

RESULTS

In vitro analysis

Mitochondrial content in differentiating NSCs in vitro

By immunofluorescence and confocal analysis, the area covered by the mitochondrial marker TOMM20 in MAP2 positive neuronal cells was evaluated.

Graph in Figure 1 shows the quantification of mitochondrial area (i.e. TOMM20+ area) per MAP2+ dendrite in differentiating neurons showing that α 5m supplementation is capable of increasing the mitochondrial content at a 0.5% concentration.

Oxidative metabolism in differentiating NSCs in vitro

The oxidative metabolism of NSC differentiating cells was evaluated by analysing the oxygen consumption rate (OCR) of control and α 5m-treated (0.5% concentration) men-Neurons and SVZ-Neurons using the Seahorse technology.

A statistically significant increase in the OCR was observed in differentiating neurons supplemented with the α 5m composition (Figure 2A) and, accordingly, an increase of the basal and maximal respiration as shown in Figure 2B and C.

Of note ATP production was doubled (men-Neurons: from 125.1 to 280.1 pmol/min/ug, Figure 2D; SVZ-Neurons: from 156.1 to 523.0 pmol/min/ug) indicating higher energy disposal.

In line with the previous results, therefore, an increase in mitochondrial functionality was observed for the cells supplemented with the composition of the instant application.

Dendritic arborization of NSC differentiating cells

Men-Neurons and SVZ-Neurons showed an increase in oxidative metabolism and mitochondrial activity after $\alpha 5m$ supplementation. To compare the meningeal- and SVZ-derived-NSC neural maturation in control and supplemented conditions, neurospheres were induced to differentiate into neurons and the morphological changes were analysed by immunofluorescence and confocal analysis for the neuronal marker MAP2, at 10 DIV.

While $\alpha 5m$ supplementation did not change the percentage of MAP2⁺ differentiating cells, a significant increase in the mature neuronal morphology, including dendritic branching and dendritic spines were observed (Fig. 3 and Fig. 4).

When analysing the morphology of the ramified MAP2⁺ cells, a statistical increase of the mean number of branches was observed (i.e., the MAP2⁺ segment comprised between two junction points) / cell in 0.5%-supplemented men-Neurons (from 15.07 to 18.55 branches, Fig. 3A), and a similar trend was observed also in 0.5%-supplemented SVZ-Neurons (from 15.27 to 17.07 branches).

The increase in mean number of branches was in line with the statistical increase of the total dendritic length, in both populations, when supplemented with the $\alpha 5m$ composition (men-Neurons: 301.6 to 458.6 μm Fig. 3B; SVZ-Neurons: 225.5 to 285.2 μm). These data suggest an increase in the cell complexity after $\alpha 5m$ supplementation, and an increased coverage of dendrites.

In order to further characterize the increase in neuronal maturation in $\alpha 5m$ supplemented neurons, the dendritic spine number and density for each experimental condition using ImageJ software was evaluated. The dendritic spine number progressively increases in 0.5% supplemented neurons (men-Neurons: from 119.6 to 198.2 spines/MAP2⁺ neuron Fig. 4A; SVZ-Neurons 109.2 to 152.0 spines/MAP2⁺ neuron). The specific spine morphology was analysed by using the Neuron Studio software (Rodriguez et al., 2008). Dendritic spines of mature

neurons modify the morphology from elongated thin spine to more stable stubby and mushroom spines.

In men-Neurons an increase in stubby spines was observed at 0.1% and 0.5% concentration of $\alpha 5m$ composition (from 36.15% to 48.62% and 47.56%, respectively) and in SVZ-Neurons an increase in the fraction of mushroom spines at 0.1% and 0.5% (from 22.81% to 28.58% and 29.03%, respectively).

These data suggest that dendritic spines in both men-Neurons and SVZ-Neurons supplemented with the $\alpha 5m$ composition acquire a stable morphology (i.e. stubby and mushroom, respectively) confirming the effect of $\alpha 5m$ supplementation in enhancing neuronal maturation.

To confirm the maturation of the differentiating neurons, the presence of synapses was assessed by analysing the expression of the pre- and post-synaptic excitatory markers Synaptophysin and PSD95, in control and in $\alpha 5m$ -supplemented neurons using ImageJ software (Dzyubenko et al., 2016).

The number of Synaptophysin immunoreactive puncta (Fig. 4B), as well as the number of PSD95 immunoreactive puncta (Fig. 4C), increased in supplemented neurons compared to control.

These results confirm the increased maturation in $\alpha 5m$ supplemented neurons compared to the control, as they show more branches, more mature spines and more synaptic markers, due to an enhancement of the oxidative metabolism in differentiating neurons.

In vivo analysis

Adult female C57BL/6J mice were exposed to laminectomy and the contusive injury was performed at vertebrate thoracic level 11 (T11). The procedure of severe SCI, due to the severity of the performed surgeries or to complication during the recovery period caused the death of 1 animal out of 50.

Supplementation of the $\alpha 5m$ composition (3g/day in the drinking water) was administered three days post SCI (3dpi). This therapeutic approach starts approximately at the end of the sub-acute phase of SCI pathogenesis. The supplementation was maintained for 31 days. Locomotor recovery and tissue histology of treated and control animals were compared.

Locomotor evaluation

In order to evaluate the effects of the $\alpha 5m$ supplementation on the motor functional recovery after a severe SCI, the Basso Mouse Scale (BMS) analysis was performed. The locomotor performance of each mouse was assessed at 1, 3,

5, 7 dpi during the first week after the SCI surgery and twice a week (10, 14, 17, 21, 28, 31 dpi) till the end of the experiment (31 dpi), when mice were perfused and spinal cords were dissected. Only mice that show a BMS score of “0” at 1 dpi were included in the analysis; mice with higher BMS score were excluded from the study.

The graph of Fig. 6 represents the BMS score of α 5m composition treated (line above with triangles) and control (line below with squares) after severe SCI. The arrow indicates the starting day of α 5m composition administration (3 dpi). Injured mice received α 5m composition in sterile water (n=22 among 3 experiments) or only sterile water (n=25 among 3 experiments).

The BMS of treated mice gradually and significantly increase compared with control SCI mice over the 28 days of administration.

At 31 dpi treated mice reached a mean BMS score of 1.773 ± 0.2434 while the control group reached a mean BMS score of 0.92 ± 1.772 (differences between experimental conditions were analysed with 2way ANOVA test and post-hoc Tukey post-test).

Therefore, mice treated with α 5m composition showed significant improvement in locomotor recovery compared to control mice. As showed in Figure 6, Post-hoc analysis revealed significant group differences in the BMS score at 14, 17, 21, 24, 28, 31 dpi.

Ankle movement analysis

‘Spasticity’ is understood to be a common symptom resulting from an injury (Adam and Hick, 2005) and it is a major cause of disability in individuals affected by a variety of central nervous system diseases (such as multiple sclerosis, stroke, and cerebral palsy) and trauma (including SCI and brain injury).

In order to determine if the supplementation with the α 5m composition has an effect in avoiding the instauration of a spastic condition of the muscles, the degree of movement of the ankle joint was evaluated. The spasticity of each treated and control mouse was assessed daily starting from 21 dpi till the end of the experiment (31 dpi), Figure 7.

The ankle joint flexibility of treated animals show a significant improvement compared to the control one. At 31 dpi, the ankle movement exhibited by treated animals was 0.6888 ± 0.0455 , while for the control was 0.5939 ± 0.0651 (differences between experimental conditions were analysed with 2 way ANOVA test and post-hoc Tukey post test).

Mice treated with $\alpha 5m$ composition therefore showed a significantly higher ankle movement compared to control mice from 21 dpi until 29 dpi indicating a faster recovery from muscles spasticity after injury.

Bladder functionality evaluation

5 One of the most common effects after SCI is the dysfunction of the bladder activity. Following SCI the bladder, usually referred as neurogenic bladder, undergoes dramatic changes that result in a spastic bladder or a flaccid one (Taweel W.A et al., 2015). In order to investigate if, after severe SCI, the administration of $\alpha 5m$ composition could exert a beneficial role in the recovery of
10 the bladder functionality, the daily diuresis of $\alpha 5m$ composition treated and control mice was analysed.

$\alpha 5m$ composition treated and control animals during the experiment showed a similar daily diuresis (Figure 8). At 31 dpi treated group showed a mean daily diuresis of $0.6359g \pm 0.1073g$ while control group of $0.5889g \pm 0.1145g$ thus
15 providing the evidence of the effect of the $\alpha 5m$ composition in the recovery of the functionality of the bladder after injury.

Tissue histology

Traumatic insults to the spinal cord lead to mechanical damage and subsequent tissue degeneration. These events are associated with shearing of cell
20 membranes and axons, disruption of the blood-spinal cord barrier, myelin degradation, immune cell transmigration and spinal cord cell death (Zhang et al., 2012). In order to investigate how $\alpha 5m$ composition treatment contributes to the regeneration of the injured tissue, spinal cord parenchyma cystic (cyst) and glial scar formation, neuronal number, myelin content.

Cystic and glial scar formation

25 After SCI the formation of a glial scar and a cystic formation (Hu R et al., 2010, Yi-Min Yuan and Cheng He, 2013) around the injury site contribute to limit the lesioned area. However, it inhibits the axonal regeneration after a SCI (Rooney GE et al., 2009) acting as a physical and molecular barrier (Fehlings M.G. and Tator C.H., 1999).
30

Considering the importance of the glial cell response to the injury, the Inventor of the instant application evaluated if the administration of the $\alpha 5m$ composition had a role on the scar formation and astrocyte reaction. Immunofluorescence staining using the specific marker for astrocytes, Glial
35 Fibrillary Acidic Protein (GFAP) was performed. Spinal cord transversal sections

covering a parenchyma segment of 1.5 cm length (15 transversal spinal cord slices/animal) centered at the lesion site (lesion performed at T11 vertebra level) was observed.

Fig. 9 shows transversal spinal cord slices immunostained with the specific astrocytic marker GFAP and TO-PRO3TM of a control (A, C) and a α 5m composition treated mouse (B, D) illustrating the regions considered for the quantifications. The grey dashed area indicates the glial scar area. The white dashed area indicates the cystic (cyst) area. As shown in panel E, the administration of the composition herein disclosed led to a significant reduction of the cyst area ($21.62 \pm 2.505\%$) compared to the control ($42.94 \pm 3.249\%$). Panel F shows that, following the α 5m administration, animals showed a significant larger glial scar area ($78.81 \pm 2.232\%$) compared to the control ($57.06 \pm 3.294\%$).

Fig. 10 shows a graph representing the percentage ratio of the glial scar area on the total slice area in control and α 5m treated mouse. Following α 5m treatment, animals showed a significant smaller cyst area ($3.55 \pm 0.6756\%$) compared to the control animals ($7.2 \pm 0.7068\%$).

These data highlight the high efficacy of the composition herein disclosed in counteracting neural tissue loss after injury.

Neuronal content

The spinal cord tissue degeneration due to the first mechanical injury is followed by a secondary insult that determines further destruction of neuronal cells (Zhang N. et al., 2012).

In order to investigate the effects of the supplementation with the α 5m composition on the neuronal content after a severe SCI, the Inventor assessed the number of neurons in spinal cord lesioned parenchyma by immunofluorescence staining with the specific neuronal marker NeuN. Spinal cord transversal sections were analysed, covering a parenchyma segment of 1.5 cm length (15 transversal spinal cord slices/animal) centered at the lesion site (lesion performed at T11 vertebra level).

First of all, the number of NeuN-positive cells in the whole spinal cord slice was evaluated. Strikingly, analysis showed that in the perilesioned area, in the α 5m treated group more neurons were present (613.3 ± 24.04) than in the control group (224.9 ± 58.03) and the difference was statistically significant (Fig. 11).

The Inventor also evaluated if the difference in the neuronal content was

maintained also in the ventral area of the spinal cord slices, where motor neurons are located. Also in this case, the number of NeuN-positive cells resulted significantly higher in the $\alpha 5m$ treated group (255.8 ± 12.35) compared to the control group (62.66 ± 13.51). The difference was statistically significant thus providing the evidence of the effect of the compositions herein disclosed in the recovery of the neuronal content after injury.

Neurofilament analysis

The major structural proteins in mature neuron axons are neurofilaments (NFs). Their expression is closely associated with axonal growth and maintenance of neuronal homeostasis (Wang H. et al., 2012). After SCI, the neuronal loss in gray matter is accompanied by a loss of neurofilament chain and myelin proteins (Qian J. et al., 2010).

In order to investigate the presence of axonal regrowth in $\alpha 5m$ composition-treated SCI mice, the Inventor performed immunofluorescence staining using the specific marker for the neurofilament proteins, Neurofilament-200 (NF200). For the analysis, the Inventor considered spinal cord longitudinal sections covering a parenchyma area of 1.5 cm length (6 transversal spinal cord slices/animal) centered in the lesion site (lesion performed at T11 vertebra level). The graph shown in Figure 12 represents the percentage ratio of the area covered by Neurofilament-200 on the selected region of interest (ROI) in $\alpha 5m$ -treated and control mouse.

Treated group exhibits a trend of higher NF200 content ($23.56 \pm 6.441\%$) compared to the control group ($16.22 \pm 3.31\%$; Fig. 12).

These results show the capability of the $\alpha 5m$ composition in favouring axonal regrowth after injury.

Re-myelinating potential

Demyelination constitutes a significant component of the SCI pathology and contributes to loss of function (Waxman SG et al., 1989). To assess the remyelinating potential of the supplementation with the $\alpha 5m$ composition on mice after a severe SCI, Luxol Fast Blue (LFB) staining was performed. LFB allows the identification of the myelin content in the spinal cord parenchyma (blue area), from the demyelinated area (white area), as shown in Fig. 13. The analysis was carried out on spinal cord transversal sections covering a parenchyma segment of 1.5 cm length (15 transversal spinal cord slices/animal) centred at the lesion site (lesion performed at T11 vertebra level). The graph of Fig. 13 represents the

percentage of the myelin content in the dorsal column of the spinal cord sections of α 5m-treated ($39.64\% \pm 0.3677$) and control ($38.47\% \pm 0.8927$), calculated as myelin positive pixels in the dorsal column among the pixels of the total area of the dorsal column. At 31 dpi animals that received the supplementation with α 5m
5 presented a similar myelin content to control animals thus providing the evidence of the α 5m composition remyelinating potential after severe SCI.

The results provided in the instant disclosure show that the amino-acid compositions herein disclosed, comprising a combination of leucine, isoleucine, valine, threonine, lysine, with citric acid, succinic acid, and malic acid, is able to
10 enhance the oxidative metabolism in differentiating neurons, favouring axonal regrowth after injury, and counteract neural tissue loss in a SCI animal model.

From the foregoing, it emerges clearly how the compositions according to the instant disclosure are useful for the treatment of central nervous system injuries in a subject, wherein the injuries are preferably selected in the group
15 consisting of cerebral injury, spinal cord injury, peripheral nerve injury, a demyelinating disease.

REFERENCES

- Adams, M.M., and Hicks, A.L. (2005). Spasticity after spinal cord injury. *Spinal Cord* 43, 577–586.
- Basso, D.M., Fisher, L.C., Anderson, A.J., Jakeman, L.B., McTigue, D.M.,
 5 Popovich, PG. (2006) Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma*. 23(5), 635-659.
- Bifari, F., Decimo, I., Chiamulera, C., Bersan, E., Malpeli, G., Johansson, J., Lisi, V., Bonetti, B., Fumagalli, G., Pizzolo, G., et al. (2009). Novel
 10 stem/progenitor cells with neuronal differentiation potential reside in the leptomeningeal niche. *J. Cell. Mol. Med.* 13, 3195–3208.
- Bifari, F., Decimo, I., Pino, A., Llorens-Bobadilla, E., Zhao, S., Lange, C., Panuccio, G., Boeckx, B., Thienpont, B., Vinckier, S., et al. (2017). Neurogenic
 15 Integrated Neurons in the Neonatal Cortex. *Cell Stem Cell* 20
- Dzyubenko E, Rozenberg A, Hermann DM, Faissner A. et al. (2016). Colocalization of synapse marker proteins evaluated by STED-microscopy reveals patterns of neuronal synapse distribution in vitro. *J Neurosci Methods*. 273, 149-159.
- 20 Hu, R., Zhou, J., Luo, C., Lin, J., Wang, X., Li, X., Bian, X., Li, Y., Wan, Q., Yu, Y., et al. (2010). Glial scar and neuroregeneration: histological, functional, and magnetic resonance imaging analysis in chronic spinal cord injury. *J. Neurosurg. Spine* 13, 169–180.
- Qian, J., Herrera, J.J., and Narayana, P.A. (2010). Neuronal and axonal
 25 degeneration in experimental spinal cord injury: in vivo proton magnetic resonance spectroscopy and histology. *J. Neurotrauma* 27, 599–610.
- Rodriguez, A., Ehlenberger, D.B., Dickstein, D.L., Hof, P.R., and Wearne, S.L. (2008). Automated Three-Dimensional Detection and Shape Classification of Dendritic Spines from Fluorescence Microscopy Images. *PLoS One* 3, e1997
- 30 Rooney, G.E., Endo, T., Ameenuddin, S., Chen, B., Vaishya, S., Gross, L., Schiefer, T.K., Currier, B.L., Spinner, R.J., Yaszemski, M.J., et al. (2009). Importance of the vasculature in cyst formation after spinal cord injury. *J. Neurosurg. Spine* 11, 432–437.
- Tator, C.H., and Fehlings, M.G. (1999). Review of clinical trials of
 35 neuroprotection in acute spinal cord injury. *Neurosurg. Focus* 6, e8.

- Taweel, W.A., and Seyam, R. (2015). Neurogenic bladder in spinal cord injury patients. *Res. Rep. Urol.* 7, 85–99.
- Wang, H., Wu, M., Zhan, C., Ma, E., Yang, M., Yang, X., and Li, Y. (2012). Neurofilament proteins in axonal regeneration and neurodegenerative diseases. *Neural Regen. Res.* 7, 620–626.
- Waxman, S.G., Black, J.A., Kocsis, J.D., and Ritchie, J.M. (1989). Low density of sodium channels supports action potential conduction in axons of neonatal rat optic nerve. *Proc. Natl. Acad. Sci. U. S. A.* 86, 1406–1410.
- Yuan, Y.-M., and He, C. (2013). The glial scar in spinal cord injury and repair. *Neurosci. Bull.* 29, 421–435.
- Zhang, N., Yin, Y., Xu, S.-J., Wu, Y.-P., and Chen, W.-S. (2012). Inflammation & apoptosis in spinal cord injury. *Indian J. Med. Res.* 135, 287–296.
- Zheng, X., Boyer, L., Jin, M., Mertens, J., Kim, Y., Ma, L., Ma, L., Hamm, M., Gage, F.H., and Hunter, T. (2016). Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. *Elife* 5.