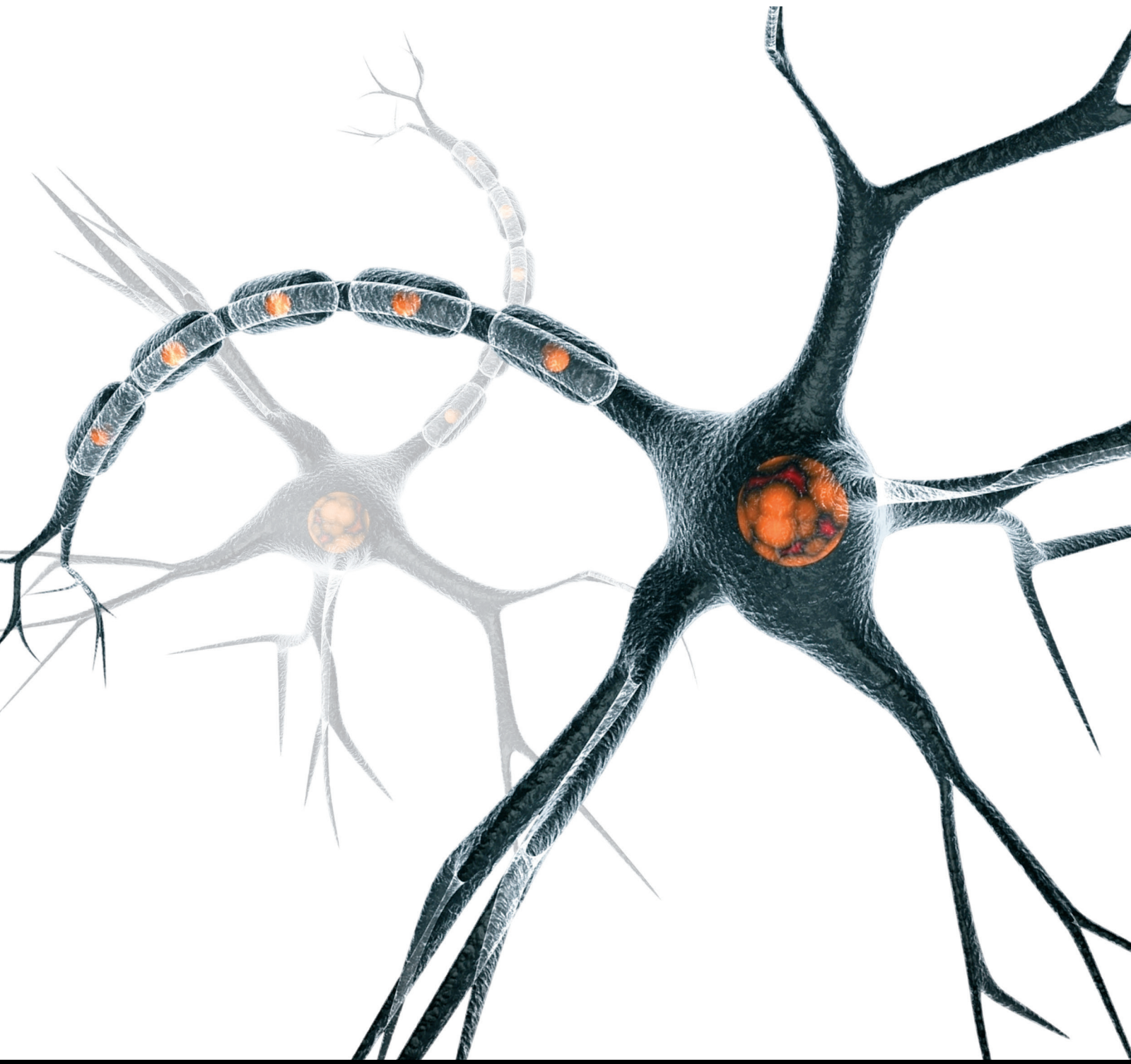
Neural Plasticity

CNS Plasticity in Injury and Disease

Guest Editors: Brandon A. Miller, John C. Gensel, and Michael S. Beattie



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Editorial Board

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 1923950, 2 pages
http://dx.doi.org/10.1155/2016/1923950

Editorial
CNS Plasticity in Injury and Disease

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Received 6 September 2015; Accepted 14 September 2015

Plasticity is a defining characteristic of the central nervous system (CNS). The ability of the CNS to physically change over the life of the organism, including myelination, neuronal proliferation, and synaptic changes, remains a topic of research in every subdiscipline of neuroscience from molecular to developmental neuroscience. The lay public also seeks a better understanding of neural plasticity. The recent BRAIN Initiative launched by the United States government (http://braininitiative.nih.gov/about.htm) aims to improve understanding of CNS plasticity by funding projects focused on neuronal circuitry, imaging, and neural modulation.

While the CNS has incredible plasticity compared to other organ systems, it also has unique sensitivity to injury. CNS injury and disease, from developmental injuries that occur in utero to environmental insults from toxins and trauma, can have a devastating effect on the organism. Ironically, some of the very mechanisms underlying CNS plasticity confer its sensitivity to injury. For example, neurotransmitter receptors that form the basis for learning and memory can be targets of excess excitatory neurotransmitters that induce cell death in many forms of CNS injury. CNS plasticity allows for recovery from injuries by both adaptation of the organism and cellular regeneration. However, these attempts at regeneration are often incomplete and unsuccessful. This was observed by the Nobel Prize winner Santiago Ramón y Cajal in the early 20th century and continues to be the basis of ongoing research on brain and spinal cord injury.

The articles in this special issue illustrate diverse examples of CNS plasticity, both in response to injury and as avenues for recovery. The scope of diseases discussed, motor neuron disease, stroke, viral infection, and spinal cord injury, illustrates that a better understanding of basic mechanisms of neural plasticity could lead to better treatments for numerous CNS diseases.

Spinal cord injury has received much attention as a target for regenerative therapies. In their paper in the issue, V. Buzoianu-Anguiano and colleagues show that a combination of degenerated peripheral nerve and bone marrow stromal cell transplantation improves axonal regrowth and myelination after spinal cord injury. Other papers in this issue address neural plasticity in diseases where it is less frequently examined. The paper by V. Atluri and colleagues highlights the neuronal injury and synaptic changes that can occur with CNS viral infection and R. Gulino reviews the plasticity that occurs in the mouse models of motor neuron disease used to model amyotrophic lateral sclerosis in humans.

Several human subject studies in this issue highlight the fact that CNS plasticity occurs in both injury and disease and that plasticity may be a target for relatively simple and potentially cost-effective therapies. A. Green and colleagues demonstrate changes in the motor cortex due to chronic spinal cord compression and G. Jiang and colleagues show that cortical changes occur after limb amputation. C. Pin-Barre and J. Laurin's review of exercise as a therapy for stroke discusses how endogenous plasticity can be enhanced by physical exercise. Similar findings from M. Weinstein and colleagues show that bimanual therapy can yield both functional and radiological changes in children with hemiparesis resulting from perinatal birth injury. These papers illustrate that treatments, including physical therapy, hold promise by increasing endogenous CNS plasticity. However, R. Brunkhorst and colleagues demonstrate that functional

improvements are not always coincident with histological recovery thereby reinforcing the complex nature of CNS plasticity.

Collectively, this special edition highlights the therapeutic and mechanistic importance of understanding and manipulating CNS plasticity in both animal models and human disorders.

Acknowledgment

We hope you enjoy this special issue that presents the evolving understanding of CNS plasticity in injury and disease.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 2769735, 11 pages
http://dx.doi.org/10.1155/2016/2769735

Review Article

Neuroplasticity and Repair in Rodent Neurotoxic Models of Spinal Motoneuron Disease

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Received 30 January 2015; Revised 12 July 2015; Accepted 19 August 2015

Academic Editor: Brandon A. Miller

Retrogradely transported toxins are widely used to set up protocols for selective lesioning of the nervous system. These methods could be collectively named "molecular neurosurgery" because they are able to destroy specific types of neurons by using targeted neurotoxins. Lectins such as ricin, volkensin, or modeccin and neuropeptide- or antibody-conjugated saporin represent the most effective toxins used for neuronal lesioning. Some of these specific neurotoxins could be used to induce selective depletion of spinal motoneurons. In this review, we extensively describe two rodent models of motoneuron degeneration induced by volkensin or cholera toxin-B saporin. In particular, we focus on the possible experimental use of these models to mimic neurodegenerative diseases, to dissect the molecular mechanisms of neuroplastic changes underlying the spontaneous functional recovery after motoneuron death, and finally to test different strategies of neural repair. The potential clinical applications of these approaches are also discussed.

1. Introduction

Motoneuron loss is the common feature of several neurodegenerative diseases, as well as mechanical injuries affecting the spinal cord (SC). Among neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) represent the most common diseases affecting spinal and brainstem motoneurons. ALS has enormous impact on the quality of life [1–3]. This disease affects mainly the lower motoneurons within the SC and brainstem, but the pyramidal neurons located in the motor cortex are also frequently damaged. This results in progressive muscle atrophy and spasticity, which ultimately cause death due to respiratory dysfunction [1, 4]. ALS is a heterogeneous disease complex that could be subdivided into two main groups: familial ALS (fALS), which accounts for only 10% of patients, and the more frequent form with no family history, affecting the remaining 90% of ALS patients, namely, the sporadic ALS (sALS) [1, 4]. The molecular mechanisms of ALS pathogenesis remain far to be fully understood and appear extremely heterogeneous. However, a number of gene mutations have been found in fALS patients, including a missense mutation in the SOD1 gene, encoding for superoxide dismutase 1 protein, which is the most frequent gene mutation found in fALS. More recently, aberrant accumulation of either mutant or wild type Tar DNA-binding protein of 43 kDa (TDP-43) has been found in both fALS and sALS, thus accounting for a common mechanism involving aberrant RNA processing and glutamate excitotoxicity [1, 4–10].

SMA is the most common inherited motoneuron disease and the main genetic cause of newborn mortality. Like ALS, SMA is characterized by the loss of spinal and bulbar motoneurons. In contrast to the multifactorial origin of ALS, this disease is unambiguously caused by the recessive mutations or deletion of the Survival Motor Neuron-1 gene (SMN1) [11–13].

A number of animal models have been developed attempting to recapitulate at least some of the genetic, anatomical, and functional defects observed in the human ALS and SMA [10, 14–17]. These models have also been used for testing

the efficacy of different repairing strategies such as rehabilitation, pharmacological, genetic, or cell-based approaches [10, 16–26].

SC injury (SCI) or nerve damage could also result in severe loss of grey matter neurons, including motoneurons [27, 28]. The mechanism of cell loss after contusion injury is complex: the mechanical damage of SC tissue (primary injury) destroys many local neurons, but it is followed by a secondary injury that kills a larger neuronal and glial population because of several pathological phenomena, including inflammation or vascular damage [29].

Although the described neurodegenerative or traumatic SC diseases are different in their etiology and pathogenesis, they share a common outcome characterized by the death of lower motoneurons. Regardless of the pathological reason for motoneuron loss, several studies have investigated the possibility of repairing the motoneuron-depleted SC by using different repairing strategies. These studies have used several animal models of selective motoneuron depletion [30, 31].

In the present paper, we performed a comprehensive review of the literature about the use of rodent models of neurotoxic spinal motoneuron degeneration, with a focus on two models obtained by intramuscular injection of volkensin or cholera toxin-B saporin (CTB-Sap). In particular, the experimental applications of these models to mimic neurodegenerative diseases, to dissect the molecular mechanisms of neuroplastic changes underlying the functional recovery after motoneuron loss, and to evaluate the effectiveness of several strategies of neural repair are extensively discussed in comparison to the other available preclinical models of disease.

2. Rodent Neurotoxic Spinal Cord Lesion Models

The first evidences about the effects of neurotoxins on motoneurons were provided as early as fifty years ago, with some studies showing the effects of tetanus and botulinum toxins on spinal motoneurons [32–34]. Afterwards, functional neuroanatomy studies have relied on the effects of lesions to investigate the function of neural systems, and a large variety of neurotoxins has been used to destroy specific cell populations. For instance, excitotoxins such as kainic acids [35, 36] or monoamine toxins including 6-hydroxydopamine [37] have been used to produce selective lesions based on the neurotransmitter specificity, but these compounds have shown incomplete anatomical and cell-type specificity. A substantial improvement of these methods of "molecular neurosurgery" has been provided by the development of axonally transported toxins such as lectins [38–41], immunotoxins [42–45], tracer-toxins, and neuropeptide-conjugated toxins [42]. When injected into the target region, these toxins are captured by axon terminals and retrogradely transported towards the cell body, thus causing cell death by ribosome inactivation and apoptosis. Plant derived lectins are anatomically but not cell-type selective, being able to kill any neuron projecting to the injection site, by suicide retrograde transport [39–41, 46, 47]. This term refers to the uptake and axonal transport of toxins by neurons projecting to the injection site, thus causing a selective lesion based on the specific neural connection rather than cell phenotype [31, 42, 47, 48]. Conversely, immunotoxins as well as tracer- or neuropeptide-conjugated toxins are both anatomically and cell-type selective, since they are internalized by cells after specific chemical binding [30, 42, 49].

A large number of plant derived neurotoxic proteins have been isolated and characterized [50], thus showing their ability to damage eukaryotic cells by acting on ribosome and catalytically disrupting the elongation step of protein synthesis [51, 52]. These ribosome-inactivating proteins (RIPs) include ricin (from *Ricinus communis*), abrin (from *Abrus precatorius*), modeccin (from *Adenia digitata*), and volkensin (from *Adenia volkensii*) [38, 39, 50, 52]. All these RIPs are axonally transported by peripheral nerves but, among these, modeccin and volkensin are more efficient to kill neurons of the central nervous system (CNS) by suicide transport [40–42, 46, 53]. Among the above described RIPs, volkensin [39] appeared to be the most toxic on CNS neurons and it has been the most frequently used to create animal models of spinal motoneuron degeneration. As early as in 1992, Nógrádi and Vrbová used volkensin with the aim of creating a reliable model of motoneuron degeneration [31]. Similar long-term effects of volkensin on the SC results were shown by Leanza and Stanzani (1998) after intramuscular injection of 2.0 ng of this RIP in newborn rats [54]. These authors have reported an extensive and long-lasting depletion of spinal motoneurons (about 90%) as measured at either two or eight months after the lesion. Afterwards, this rodent model was used, also by our research group, either as recipients in experimental approaches of transplant-induced regeneration (see Section 4) [55–57] or as models for testing the intrinsic potential for spontaneous regeneration (see Section 3) [58].

A substantial improvement of neurotoxic lesion protocols came from the development of targeted RIPs by conjugation with a specific carrier, such as an antibody, a neuropeptide, or a retrograde tracer [30, 42, 44, 45, 49, 50, 59]. Saporin, an RIP from *Saponaria officinalis* [50], is the most used toxin to prepare targeted neurotoxins. Cholera toxin is the bacterial protein toxin of *Vibrio cholerae*. It is composed of a catalytically active A subunit linked with a B subunit. The latter is responsible for the specific binding to the GM1 membrane receptor, internalization, and retrograde transport [60, 61]. Given these properties, cholera toxin-B subunit could be used either as a retrograde tracer [30, 62] or as a targeted neurotoxin after conjugation with saporin [30]. A number of *in vivo* experiments have used cholera toxin-B saporin (CTB-Sap) and demonstrated its effectiveness in removing any neuron expressing GM1 ganglioside [30, 49, 63–65]. Recently, our group has developed a mouse model of lumbar SC motoneuron degeneration by injection of CTB-Sap into the gastrocnemius muscle. The toxin has been injected into the medial and lateral gastrocnemius muscles at a dose of 3.0 μg/muscle and caused a partial depletion of lumbar motoneuron (25–30%), accompanied by an evident impairment of the hindlimb motor function [66]. Given the moderate severity of the lesion, this model is suitable for evaluating the spontaneous recovery of locomotion and the

underlying SC plastic changes, such as neurogenesis [66] or synaptic plasticity (see Section 3) [66–69].

3. Mechanisms of Spinal Cord Plasticity in Models of Motoneuron Disease

Several evidences have demonstrated that adult mammals could achieve a significant range of spontaneous sensory-motor recovery after injury or disease, by means of various forms of neuroplasticity. This plasticity includes the recruitment of neural precursor cells (NPCs) and the formation of new pathways as well as synaptic plasticity, within the affected tissue and/or in sensory and supraspinal pathways [70–73]. However, this spontaneous plastic potential is inadequate for allowing complete regeneration and recovery of function, but some therapeutic interventions are able to recruit and potentiate this intrinsic capacity, thus producing a better outcome. Since it has been found that SC plasticity is activity-dependent [74], a number of studies have demonstrated the effectiveness of exercise training and other methods of "spinal learning" in both animal models and human SCI patients [70, 75–77]. Some information is also available about plastic changes occurring in neurodegenerative diseases and, in particular, in motoneuron disease. It is known, for instance, that plastic changes could occur in Parkinson's disease [78] as well as in the respiratory system and brain of ALS patients [79–81], but the beneficial effect of exercise training is still controversial [82, 83]. Given the progressive nature of these diseases, it is obvious that any compensatory change will ultimately be ineffective. Despite these limitations, a better understanding of the plastic phenomena occurring in animal models of motoneuron disease would help in elucidating the molecular mechanisms of diseases and finding new putative targets for therapy. Anatomical rearrangement and functional compensatory changes in spinal and supraspinal circuitry have been reported in rodent models of neuronal degeneration induced by nerve crushing [84, 85].

The previously described murine model of selective CTB-Sap induced motoneuron depletion developed in our laboratory has been deeply characterized to evaluate its capacity for spontaneous sensory-motor recovery. Noteworthy, a relevant increase of motor performance measured at the grid walk or rotarod test has been observed as early as one month after toxin injection, despite a permanent though moderate motoneuron removal [66, 68, 69]. The cellular and molecular mechanisms underlying this remarkable functional recovery have been studied, including the activation of endogenous NPCs [66], the spontaneous events of synaptic plasticity [66–69], and the expression and functional roles of neurotrophic factors [67] and/or other molecular factors including cell fate determinants [66–68] and TDP-43 [69].

3.1. Neurogenesis. NPCs proliferation and differentiation take place spontaneously in the adult mammals only in the subventricular zone and hippocampus [86, 87]. However, multipotent NPCs could be isolated from the entire adult CNS, including the SC [88–90]. Several experiments have demonstrated that these cells could be mobilized after SCI but, unfortunately, they only generate migratory cells that differentiate to astrocytes and participate in scar formation [89, 91, 92]. Notably, astrocyte activation could also be caused by a selective neurotoxic neuron removal by volkensin suicide transport in either brain or SC [31, 93]. Moreover, a significant amount of cell proliferation and increase of GFAP-positive astrocytes have been found in the SC ventral horn, after selective motoneuron removal by intramuscular injection of CTB-Sap [66]. Glial reaction is a classical response to CNS tissue damage, which generally also involves glial cells themselves and induces a series of events that amplifies and maintains glial activation [94, 95]. Therefore, the glial reaction observed after selective neuronal loss, with the absence of severe tissue damage and inflammation [96], could have different origin as well as different consequences on regenerative processes.

Intrinsic and extrinsic molecular factors regulating adult neurogenesis have been widely explored [86, 97]. Sonic hedgehog (Shh) is a secreted glycoprotein promoting NPCs proliferation and differentiation to neurons and oligodendrocytes, during both development and adulthood [98, 99]. The Notch-1 pathway and its inhibitor Numb are also involved in the regulation of NPCs proliferation, cell fate determination, dendritic morphology, and axon guidance in embryonic and adult CNS [100–103], including SC [104, 105]. Noggin is a secreted glycoprotein responsible for neural induction during development, by acting as an inhibitor of bone morphogenetic proteins [106]. As shown by Chen and colleagues (2005), Shh, Notch-1, and Numb expression are increased in the SC after compression injury [107]. However, unlike their embryonic counterparts, NPCs are unable to generate neurons in the adult SC. Recently, some experiments have been performed to investigate the expression and the functional role of Shh, Notch-1, Numb, and Noggin on the murine model of CTB-Sap induced motoneuron depletion [66, 68]. In contrast to those observed in SCI models, Shh and Numb expressions appear transiently decreased after motoneuron removal and then recovered in association with the spontaneous functional recovery, whereas Noggin expression progressively increases [66, 68]. The reasons for the discrepancy between mechanical and neurotoxic lesion models are elusive but some explanations could be proposed. For instance, mechanical damage affects several neuronal and glial populations, whereas the described neurotoxic lesion selectively kills motoneurons in spatially restricted regions. Moreover, ependymal cells undergo a robust proliferation immediately after a mechanical injury [89, 108], whereas they seem unresponsive in the CTB-Sap model [66] (see Figure 1).

Interestingly, a pattern of NPCs proliferation and reactive gliosis closely resembling that found in CTB-Sap models, with no evidence of neurogenesis, was found in transgenic mouse models of ALS expressing the mutated human SOD1 gene [109, 110]. Unfortunately, further information concerning these endogenous repairing potentials of ALS affected SC is still lacking, and the results provided by neurotoxic models are therefore of great importance. However, these processes need to be further clarified because they denote the importance of the role of environmental cues on the behavior of spinal NPCs. It is also likely that an experimental approach

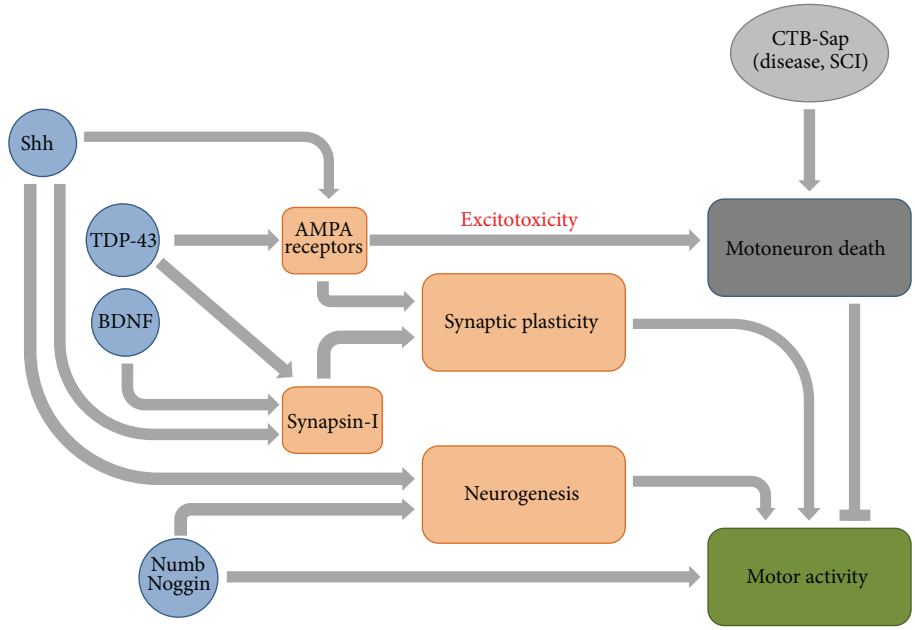

Figure 1: Proposed model of spontaneous SC plasticity after motoneuron degeneration.

aimed at artificially modifying Shh, Numb, and Noggin signaling into the SC could stimulate NPCs proliferation, reduce glial reaction, and probably drive cell differentiation towards neuronal phenotype.

3.2. Synaptic Plasticity. Another process promoting the functional restoration consists of the reorganization of spinal, supraspinal, and sensory pathways by mechanisms involving activity-dependent synaptic plasticity [71, 74, 111]. As previously described, a significant amount of spontaneous locomotor recovery is possible in rodent models of both SCI and motoneuron disease and could be driven, at least partially, by mechanisms of synaptic plasticity [66–69, 112, 113].

The molecular feature of synaptic plasticity has been extensively studied in the hippocampus, as it represents the principal mechanism underlying learning and memory. In fact, it is known that long-term modifications of synaptic efficacy are regulated presynaptically by the expression and phosphorylation of various synaptic vesicle proteins including synapsin-I [114–116] and postsynaptically by changes in the expression and trafficking of glutamate receptors. In particular, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptors are fundamental for cortical and hippocampal synaptic plasticity [117–120]. The emerging role of astrocytes and their expression of connexins in the modulation of synaptic strength are also noteworthy [121, 122].

A fundamental role in modulating both pre- and postsynaptic changes is exerted by brain-derived neurotrophic factor (BDNF) [123–125]. In fact, synapsin-I is considered as a downstream effector of BDNF [123, 125]. Moreover, it seems clear that the activity-dependent release of BDNF could regulate the synthesis and synaptic delivery of AMPA receptors in different brain areas [126, 127] and, conversely, the glutamate receptor activity could modulate BDNF release [128, 129]. Interestingly, several authors have shown that such mechanisms could take place also in the intact and lesioned SC [112, 113, 130], as well as in the mouse model of CTB-Sap induced motoneuron loss developed in our laboratory. In particular, we have found that the spontaneous recovery of locomotion observed in the motoneuron-depleted mice is linked to the expression levels of both synapsin-I and AMPA receptors [66–68]. Moreover, this model has confirmed the described role of BDNF [67] and has also provided evidence about novel functional roles of Shh, Numb, and Noggin that, in addition to the traditional role as cell fate determinants, could also participate in modulating synaptic plasticity and functional recovery [66–69] (see Figure 1).

Information about the occurrence of synaptic plasticity in patients or animal models of ALS is poor. However, it is noteworthy that the expression of synaptic vesicle proteins is significantly decreased in the SC ventral horn of ALS patients [131], thus again confirming that CTB-Sap models could be interesting research tools for research in motoneuron disease.

3.3. The Emerging Role of TDP-43. TDP-43 is a nuclear DNA/RNA-binding protein encoded by a highly conserved gene and involved in mRNA processing [132, 133]. Recently, TDP-43 was found in the cytoplasmic protein aggregates observed in some neurons of patients affected by ALS [6, 133]. Therefore, increasing attention has been devoted to the toxic effects of mutant TDP-43 on motoneurons but, more recently, it is becoming likely that some of these effects could depend on the loss of function of the normal TDP-43 [5, 7, 133, 134]. In addition to the described classical role, TDP-43 could be involved in apoptosis, microRNA biogenesis, and cell proliferation [132]. Notably, TDP-43 has been found in the dendrites, where it could affect local RNA translation in an

activity-dependent manner [135, 136]. Moreover, TDP-43 is crucial for synaptic formation and plasticity, as well as for locomotion in *Drosophila* [134, 137, 138].

It has been recently shown in our model of motoneuron loss that synapsin-I expression is linked to that of TDP-43 and that the latter correlates with the expression of AMPA receptor subunits GluR1, GluR2, and GluR4 [69]. This association is interesting. As mentioned above, synaptic plasticity is modulated by AMPA receptor trafficking and in particular by the regulation of Ca^{2+}-permeable AMPA receptors [117–119]. The ion permeation is linked to the amount of Q/R-unedited GluR2 subunits included into the AMPA channels. Therefore, given that TDP-43 is likely involved in the Q/R-editing of GluR2 subunits, one of the proposed mechanisms of motoneuron death in ALS is the glutamate toxicity caused by the aberrant increase of unedited GluR2 subunits [5, 8]. Similar processes could take place in the CTB-Sap SC lesion model and, interestingly, the same events could affect synaptic plasticity in this model. Unlike the functional linkage between AMPA receptors and TDP-43, the association between synapsin-I and TDP-43 is absolutely novel and suggests a model where TDP-43 could affect synaptic strength by modulating the expression of both synapsin-I and AMPA receptors [69] (see Figure 1). This hypothesis is supported by other evidences that TDP-43 is present at synapses and controls the local synthesis of synaptic proteins [135, 139]. Other recent findings have shown that the lack of TDP-43 could affect synapses and cause locomotor deficits in *Drosophila* [134, 137].

Given the increasing interest in mouse models of TDP-43 gain or loss of function as models of neurodegenerative diseases, including ALS [10, 16, 140], is likely that the elucidation of the physiological role of TDP-43 in the SC would provide an important contribution.

4. Repairing Strategies

To date, neurodegenerative disorders such as ALS and SMA do not benefit from any effective therapy. Riluzole represents the only approved therapy for ALS, but its effects consist in prolonging survival and delaying the use of supportive care by a few months [141]. As previously discussed, the adult SC is capable of a significant amount of spontaneous functional restoration, and this is particularly evident in rodent models of SC injury or disease [66, 68, 71, 74, 111]. Although this capacity is not enough to allow full recovery, it is anyway encouraging because the elucidation of the underlying cellular and molecular mechanisms would provide novel therapeutic tools and targets, thus improving the expected clinical outcomes. As the spontaneous functional recovery could be driven by the recruitment of NPCs, regeneration of damaged neurons, and events of synaptic plasticity occurring within the spared circuitries, the improvement of these processes by external interventions would represent effective therapeutic strategies. Several preclinical studies have shown that cell-based therapies could also be promising. However, further studies employing representative preclinical models, as well as the design of clinical trials, are mandatory to make this increasing knowledge available for translational applications.

4.1. Non-Cell-Based Therapies. The activity-dependent nature of plastic changes within the SC [71, 74] has suggested the possibility that the damaged SC could be retrained in an attempt to modify the activity of the spared circuitries and compensate for the partial loss of neurons and connections [142]. Several animal models of SCI have been used so far to test this hypothesis. Locomotor training has proven to be beneficial in spinalized animals [76, 112, 113], by mechanisms of activity-dependent BDNF-induced synaptic plasticity [112, 113, 130, 143]. Significant clinical improvement could also be achieved by human SCI patients as a result of locomotor training [70, 75]. The importance of plastic changes in motoneuron diseases needs further investigations and the data provided by neurotoxic models would also be helpful as previously discussed. Few studies have investigated the therapeutic value of exercise training in either human patients or animal models of motoneuron disease and produced controversial findings. A couple of studies involving SOD1 mouse models demonstrated that a moderate exercise could produce neuroprotective effects on motoneurons, although the impact on the life span is controversial [144–146]. Moreover, the beneficial effects seem to be dependent on the type of physical exercise [18]. Similar results have been provided by a small number of studies involving human patients [82, 83], thus indicating that further studies are needed to clarify the relationships among neuronal activity, motoneuron vulnerability, and neuroprotection. In this respect, important insights have been provided by the previously described CTB-Sap SC lesion model (see Section 3) [66–69], but some of them require further investigation and clinical trials. In particular, the role of neurotrophins and other growth factors has been confirmed in different animal models including the CTB-Sap lesioned and the other established animal models of disease [17, 147]. However, human trials showed inconsistent or negative effects of growth factors due to different reasons such as bioavailability, poor penetration through the blood-brain barrier, and inadequate or excessive dosing.

Other studies for effective treatments have focused on the neuromuscular junction and the role of the skeletal muscle as source of chemical and cellular cues sustaining neuronal survival, axonal growth, and synaptic connections, such as trophic support or the role on Nogo-A [19]. The CTB-Sap model could help in investigating this aspect without unwanted environmental cues, which are normally present in the genetic models of ALS or SMA.

4.2. Cell Therapy. Cell transplantation was one of the first repairing approaches used in models of SC injury and disease. Transplantation of fetal motoneurons was successfully used in models of motoneuron loss induced by nerve crushing [148], kainic acid [149], or volkensin [55–57, 150] and demonstrated that the grafts were able to survive and develop as functionally active mature motoneurons [55–57, 150], although their capacity of muscle reinnervation was limited. More recently, cell-based strategies have relied on the potential beneficial effects of stem cells such as embryonic, neural, mesenchymal, and induced pluripotent stem cells [19–26, 151–153]. A number of preclinical studies have proven that stem cell therapy is able to delay the disease progression,

rescue motoneuron function, and extend survival in animal models of ALS or SMA. Multiple mechanisms are responsible for these beneficial effects. It is obvious, for instance, that replacement of lost motoneurons is an important goal in repairing strategies, but some limitations still occur as previously described, including integration into the host tissue and reinnervation. Moreover, resident as well as grafted neurons could be susceptible to degeneration if exposed to a toxic microenvironment like that present within the diseased neural tissue. Transplantation of cells including different stem cell types could provide trophic support, remove toxic cues, and exert immunomodulatory effects, which ultimately could result in neuroprotection for motoneurons [19–26, 151–153]. The use of a neurotoxic model, where motoneuron depletion is not accompanied by a chronic disease state or toxic environment, could offer a different point of view for elucidating the beneficial effects of cell-based therapies. A number of stem cell clinical trials [19, 154] have shown that some cell-based protocols could be safe and produce promising though modest effects. Regarding the cell source, mesenchymal stem cells could be easily obtained from patients and are considered suitable for autologous transplantation. Interestingly, induced pluripotent stem cells represent a novel source for autologous stem cells. They can be obtained by reprogramming somatic cells without viral methods and differentiated towards multiple phenotypes [19–26, 151–154]. However, to achieve effective cell-based therapies suitable for clinical application, several issues should be addressed, including the optimization of delivery protocols (route of administration, dose) and the better elucidation of the graft-host interaction. The ideal route of administration should produce the best therapeutic effects with the minimal invasiveness. Intrathecal or intravenous administration could represent effective approaches, because they ensure the widespread distribution of cells, which is ideal when degeneration is not limited to a small area. However, cells must be able to penetrate the blood-brain barrier and migrate correctly towards the affected areas. Again, several preclinical studies are needed, by using different animal models, to address these important goals.

4.3. Recruitment of Endogenous Neurogenesis. As previously discussed, NPCs proliferation occurs in different animal models of motoneuron loss, including neurotoxic and ALS models, but external interventions are needed to potentiate this capacity and drive NPCs differentiation towards the neuronal phenotype [66, 109, 110]. Bambakidis and colleagues (2003) have treated SC lesioned rats with Shh and provided evidence of increased NPCs proliferation and their differentiation as oligodendrocytes and neurons [73, 155]. In addition, Shh promotes survival and exerts neuroprotective effects on CNS neurons including motoneurons [156, 157]. A recent study showed that G93A mouse model of ALS produced spontaneous NPCs proliferation within SC lamina X, which was increased by lithium administration. Moreover, lithium-treated animals showed increased neuronal differentiation and attenuation of disease progression [110]. Another growth factor not only stimulating neurogenesis but also promoting neuronal survival, migration, and axon guidance in ALS models as well as protection of motoneurons against excitotoxicity is Vascular Endothelial Growth Factor (VEGF) [158, 159]. Beneficial effects of many other growth factors and morphogens, as well as hormones, on SC repair have been published by several authors. Axonal growth and other plastic changes could be promoted, for instance, by Noggin and BDNF [113, 160–162], whereas testosterone treatment has proven to exert neuroprotective effects on motoneurons in CTB-Sap lesion models, by preventing dendritic atrophy after removal of surrounding motoneuron [63, 65].

5. Concluding Remarks

Further studies are needed to better understand the mechanism of neurodegeneration as well as develop effective methods of therapy and rehabilitation. In this respect, although a large number of studies will be obviously conducted on mouse models of ALS and SMA, the above-described neurotoxic models of motoneuron degeneration will certainly be useful as well. In fact, these models are easy to be produced and characterized. Moreover, motoneuron-depleted SC is a simple and powerful tool for cell transplantation and for testing plastic changes and the consequent functional outcome. Despite the difference between neurotoxic and genetic rodent models, the described similar effects on neurogenesis and the involvement of TDP-43 and the multiple roles of neurotrophins and morphogens would open a number of novel research pathways aimed at the dissection of pathogenesis and selection of new therapeutic targets and tools for the treatment of motoneuron diseases.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 608581, 12 pages
http://dx.doi.org/10.1155/2015/608581

Review Article

Physical Exercise as a Diagnostic, Rehabilitation, and Preventive Tool: Influence on Neuroplasticity and Motor Recovery after Stroke

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Received 30 January 2015; Revised 3 June 2015; Accepted 18 June 2015

Academic Editor: Brandon A. Miller

Stroke remains a leading cause of adult motor disabilities in the world and accounts for the greatest number of hospitalizations for neurological disease. Stroke treatments/therapies need to promote neuroplasticity to improve motor function. Physical exercise is considered as a major candidate for ultimately promoting neural plasticity and could be used for different purposes in human and animal experiments. First, acute exercise could be used as a diagnostic tool to understand new neural mechanisms underlying stroke physiopathology. Indeed, better knowledge of stroke mechanisms that affect movements is crucial for enhancing treatment/rehabilitation effectiveness. Secondly, it is well established that physical exercise training is advised as an effective rehabilitation tool. Indeed, it reduces inflammatory processes and apoptotic marker expression, promotes brain angiogenesis and expression of some growth factors, and improves the activation of affected muscles during exercise. Nevertheless, exercise training might also aggravate sensorimotor deficits and brain injury depending on the chosen exercise parameters. For the last few years, physical training has been combined with pharmacological treatments to accentuate and/or accelerate beneficial neural and motor effects. Finally, physical exercise might also be considered as a major nonpharmacological preventive strategy that provides neuroprotective effects reducing adverse effects of brain ischemia. Therefore, prestroke regular physical activity may also decrease the motor outcome severity of stroke.

1. Introduction

Despite progress in functional rehabilitation, stroke patients frequently present chronic motor disabilities [1]. Therefore, it seems crucial that both scientists and therapists continue to investigate stroke physiopathology and improve the effectiveness of physical training programs on motor recovery. In recent years, physical exercise was used in stroke experiments for 3 main purposes, namely, the detection of physical dysfunctions, the improvement of motor activity, and the prevention of severe damage. Such contributions might ultimately improve both physical independence and quality of life, while reducing cardiovascular complications and recurrent stroke [2, 3].

First, acute fatiguing exercise is used in preclinical experiments as a diagnostic tool to detect sensorimotor dysfunctions and/or reveal treatment effectiveness that cannot be observed in resting condition. Indeed, alteration of motor unit activation in both affected and unaffected sides or changes of the motor reflex regulation were highlighted during/after acute exercise [4, 5]. Moreover, a higher corticospinal tract activity was found only after acute treadmill exercise in trained patients contrary to untrained patients [6].

Then, added to its beneficial effects on cardiorespiratory fitness and muscular endurance, chronic physical activity is effective as a rehabilitation tool for improving functional recovery and promoting neural plasticity [3, 7]. Indeed, it

was recently found that physical training promoted cerebral angiogenesis, vasomotor reactivity, and neurotrophic factor release but also reduced apoptosis processes, excitotoxicity, and inflammation into the peri-infarct site and could improve the regulation of motor unit activation [8–12].

Nevertheless, physical training seems to remain insufficient to completely restore neural and motor functions. A recent approach for stroke therapy is to combine physical training with pharmacological treatments, known to accentuate and/or accelerate neuroplasticity. In the present review, we discuss the influence of physical training with or without additional pharmacological treatment on neuroplasticity and motor recovery after stroke.

Although regular exercise reduces the risk of developing stroke [13], it may occur for physically active individuals [14, 15]. However, endogenous neuroprotective effects induced by prestroke physical activity may exhibit beneficial influence on recovery by reducing both brain damage and motor outcome severity [16–19].

The present review is designed to discuss the multiple uses of physical exercise to improve neural and motor recovery following cerebral ischemia/stroke from both animal and human studies. We suggested that exercise-induced neural plasticity is crucial to better understanding motor outcomes after stroke and improving rehabilitation program effectiveness.

2. Acute Exercise-Induced Neural Adjustments after Stroke

Animal and human studies reported that acute exercise could reveal changes in muscle activation regulation after cerebral ischemia. More precisely, the electromyographic (EMG) activity was lower in affected hindlimb muscles during a single bout of treadmill exercise compared to unaffected muscles, reflecting a strong decrease of the motoneuronal recruitment from spinal and/or supraspinal motor pathways [4, 20]. A reduced corticospinal excitability to the paretic quadriceps was also observed by showing strong decrease of the motor evoked potential (MEP) amplitude during a running exercise [4]. In addition, cortical activation increased in the unaffected side while it decreased in the affected side after ankle dorsiflexion movements that suggested compensatory neural mechanisms [21–26].

Spinal motor reflex plasticity also seems to contribute to motor disorders after stroke but the underlying neural mechanisms remain poorly understood [36, 37]. A recent study has demonstrated that the motor reflex regulation (H reflex) was acutely altered following an exhaustive isometric exercise on affected triceps brachii in MCAO rats whereas resting H reflex did not differ between injured and noninjured animals [5]. It was postulated that such findings might be partially due to an alteration of motor reflex regulation by muscle afferents that remained activated after intense exercise (groups III and IV muscle afferents).

Fatigue process alterations could also be observed during dynamic exercise after cerebral ischemia by using EMG recording. When fatigue progresses, there is a shift to lower motor unit frequencies. Median power frequency (MPF), which is the sum of product of the EMG power spectrum and the frequency divided by the total sum of the power spectrum, is frequently used for the assessment of muscle fatigue in surface EMG signals [38]. Therefore, a decrease in MPF serves as an index of fatigue [39]. After cerebral ischemia, the observed MPF drop at the unaffected hindlimb reflected a higher muscle fatigue compared to the affected one (no MPF decrement). A lesser fatigue-related decrease in median frequency was also observed in humans at the paretic side compared to the nonparetic side during both voluntary contractions and locomotor activity [20, 40]. This accelerated fatigue process of the unaffected hindlimb was usually explained by its higher weight bearing that compensated for the affected side. Nevertheless, reduction of work without MPF decrease of affected muscle was reported during repeated eccentric-concentric actions suggesting that peripheral fatigue through excitation-contraction coupling disruption might contribute to exercise intolerance and neuromuscular disorders of stroke patients [41].

It was also found that acute exercise-induced neural adjustments could reveal the effectiveness of a given treatment. Indeed, Forrester and colleagues have first compared trained and untrained stroke patients on the observed neural response to a single bout of treadmill exercise [6]. They found that the MEP amplitude increased in trained patients after this exercise contrary to untrained individuals, in which MEP amplitude remained unchanged. Such training corticospinal adaptation was not observed in the preexercise MEP amplitudes, but only after exercise, and provided insight into the effectiveness of 6-month treadmill training [6].

3. Effects of Poststroke Physical Training on Neuroplasticity and Motor Recovery

The effects of chronic physical training in stroke patients have received more attention in scientific literature than the ones of acute exercise. Indeed, physical rehabilitation remains the first-line intervention strategy to attenuate chronic impairments of sensorimotor function by promoting brain organization and reducing infarct volume during the first weeks after stroke [42, 43]. Given that the infarct size was not always correlated with motor recovery, it was suggested that other adaptive mechanisms might take part [44, 45]. Numerous recent studies indicated that early treadmill training promotes neuroplasticity by acting on brain vasomotor activity and angiogenesis, neurotrophic factor and apoptosis marker expressions, brain inflammatory processes, blood brain barrier (BBB) integrity, and muscle activation control (Figure 1) [46–48].

Cerebral blood flow in the ischemic region is affected following human stroke and cerebral ischemia in mice due to impaired cerebral vasomotor reactivity [8, 49]. Therefore, restoring an adequate cerebral vasomotor reserve capacity is crucial to supplying required nutriments and O_2, as well as reducing infarct volume and functional deficits [50, 51]. Preclinical and clinical studies bring strong evidence on endurance training effectiveness to promote vasomotor reactivity (endothelium-dependent vasorelaxation) and angiogenesis in the ischemic penumbra, which contribute to

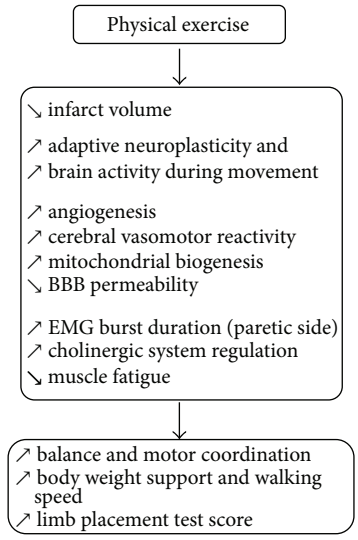

Figure 1: Beneficial structural and functional plasticity induced by physical exercise in stroke individuals.

limiting brain damage and motor deficits [9, 50]. Indeed, treadmill training increased the most commonly studied angiogenic growth factor expression, namely, vascular endothelial growth factor (VEGF), and its regulatory protein, caveolin-1, that might contribute to explaining the increase of new vessel growth and vascular density [52–56]. The training effect on angiogenesis was reinforced by evidence showing that 2 weeks of treadmill training increased the angiopoietin expression, another angiogenic growth factor that has a key role in new vessel formation [54]. Other findings revealed that the area of platelet-endothelial cell adhesion molecule- (PECAM-1-) immunopositive cells (protein involved in angiogenesis and integrin activation) was significantly increased around the infarct after 28 days of treadmill training [46]. Daily treadmill training induced an increase of GFAP expression (proteins playing a role in vascular cerebral plasticity) suggesting that area of neovascularization was higher in exercised animals [47]. Moreover, voluntary running training upregulated by 3- to 4-fold aortic endothelial nitric oxide synthase (eNOS) mRNA expression and it remained elevated 10 days after training [8, 9]. Such findings concur with the fact that beneficial effect of running was completely abolished in animals lacking eNOS expression and in mice treated with a NOS inhibitor or an antiangiogenic compound such as endostatin [9]. Moreover, training program increased endothelial progenitor cells (EPC) in bone marrow that are known to strongly influence eNOS expression. It is noteworthy to add that only 3 days of aerobic exercise reduced brain microvascular endothelial cell apoptosis related to the increase of shear stress that was followed by modest improvement of cerebral blood flow [51].

Physical training can also upregulate the expression of some neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and insulin-like growth factor (IGF-1).

BDNF is one of the most active neurotrophins that binds to the tyrosine kinase receptor (trkB). Overall, this association triggers several molecular pathways that promote neural proliferation and survival and synaptic and axonal plasticity by enhancing synapse formation, dendritic growth, and remodeling [57]. Furthermore, BDNF seems also to act on motor function because blocking BDNF mRNA expression by injecting antagonist reduced skilled motor abilities [58].

The neuroprotective effect of endogenous and exogenous BDNF is well established after brain infarction [12, 59–62]. Basal endogenous BDNF/trkB expression increased within both hemispheres following moderate cerebral ischemia (including the penumbra) during the first 2 weeks but motor recovery remains widely insufficient [63–65]. Therefore, treatments that enhance BDNF/trkB production seem relevant to improve motor recovery (even if infarct size is not modified) [11, 66]. It was found that delayed intravenous BDNF administration (20 μg/day) for 5 days improved long-term functional outcomes such as running and sensorimotor recovery after ischemia [11, 66, 67].

Interestingly, endurance training stimulated endogenous BDNF/tkrB expression and may play a neuroplastic role following cerebral ischemia or intracerebral hemorrhage in rat and mice [58, 63, 68–72]. This enhanced BDNF/tkrB was observed in both hemispheres but especially in the nonlesional hemisphere compared to healthy animals under the same program (that might represent a compensatory mechanism) [53, 69]. Despite the fact that there is no direct evidence, the training-related BDNF expression is often associated with motor recovery improvement [58, 63, 70]. It was demonstrated that 7 consecutive training days could be sufficient to increase BDNF expression and improve motor recovery [72–74]. Although endurance exercise upregulates BDNF expression in striatum and cortex, functional outcome improvement might be more related to hippocampal BDNF expression [70, 72]. Indeed, voluntary wheel exercise is known to induce substantial motor recovery associated with the highest hippocampal BDNF level [70]. Another study also found a positive correlation between motor function recovery rate and hippocampal BDNF expression after treadmill training following cerebral infarction [72].

Finally, the number of NGF-immunopositive cells, promoting cell growth and neuronal activity, was particularly increased over a widespread region around the infarct zone in trained rats [46]. NGF expression, which may be the result of heightened neuronal activity during exercise, could contribute to reducing brain damage around 4 weeks after ischemic stroke.

Physical training could reduce ischemic brain damage and motor deficits by other mechanisms such as reduction of acute inflammatory reactions and neuronal apoptosis [75]. Indeed, it was demonstrated that the neurotrophic factor midkine (MK), which could delay the process of neuronal death during the early phase after cerebral infarction, was higher expressed in the cells of the peri-infarct region after physical training program [46].

Cerebral ischemia-induced cell death is commonly attributed to necrosis and apoptosis, but it was recently found

that inappropriate autophagy might also lead to cell death [76]. However, physical training mitigated autophagosomes accumulation and attenuated apoptosis in the peri-infarct region while improving the modified neurological severity score scale. Physical training could also upregulate IGF-1 expression, which is known to attenuate autophagy and also to promote neurogenesis [75].

In addition, several studies reported that neuronal death in both the striatum and the cerebral cortex caused a degeneration of nigral dopaminergic neurons that are known to contribute to regulating motor activity [77]. However, treadmill training promoted axon regeneration of newborn striatonigral and corticonigral projection neurons in ischemic brain while improving motor function. It was thus suggested that exercise could enhance restoration of functional neural circuitry within the basal ganglia. The cerebellum plays an important role in the motor coordination, learning, and equilibrium and also seems to be involved in motor deficits following cerebral injury. Therefore, it is not surprising that treadmill training promoted both synaptogenesis and neurite outgrowth via 25-kDa synaptosomal-associated protein expression in the cerebellum. Likewise, such training also increased glial fibrillary acidic protein in the cerebellum, which plays a role in axonal growth, and improved motor coordination as observed with the rotarod test (longer time to fall from the rotating rod) [47].

Disruption of the BBB, increasing thus its permeability, is one of the major contributors to the pathogenesis of cerebral ischemia. Such event was observed when matrix metalloproteinase-9 (MMP-9) is strongly upregulated that impairs the extracellular matrix of the BBB [78, 79]. However, physical training attenuated BBB disruption as revealed by a decrease of MMP-9 expression and a parallel increase expression of MMP-9 inhibitor, the tissue inhibitor of metalloproteinase-1 (TIMP-1). Interestingly, such mechanisms might contribute to decreasing the observed neurological deficits, infarct volume, and brain edema [80].

These neuroplasticity processes require energy from mitochondria. However, cerebral ischemia induces damage of the cerebral mitochondria biogenesis, contributing to the extent of neuronal ischemic injury [81]. Nevertheless, 7-day endurance training increased mitochondrial biogenesis after cerebral ischemia by enhancing both the amount of mitochondrial DNA and the expression of numerous mitochondrial biogenesis factors such as the mitochondrial transcription factor proliferator activated receptor coactivator-1 (PGC-1), nuclear respiratory factor 1 (NRF-1) protein, and mitochondrial transcription factor A (TFAM) [82]. It was suggested that these events are involved in decreasing infarct volume and the improvement of neurological score.

Very few studies using rat model of cerebral ischemia enable better understanding of the treadmill training outcomes on muscle activation regulation. Cerebral ischemia is believed to affect synaptic activity including the cholinergic system, which leads to decreasing both neuromuscular junction and cholinergic brain synaptic transmission. It was shown that aerobic treadmill training (20 min/day, during 21 days) improved cholinergic system regulation/homeostasis by decreasing acetylcholinesterase activity (i.e., hydrolyzing the acetylcholine) and by enhancing choline acetyltransferase activity (i.e., synthesis of acetylcholine) [53]. It was thus suggested that such adaption might allow better motor limb function as indicated by improvement of the limb placement test score. Moreover, 10 days of treadmill training improved balance, functional outcome (behavioral score), and motor coordination. Indeed, the asymmetry of muscle activation pattern between affected and unaffected hindlimbs was restored because the EMG burst duration was increased at the affected side [83]. Muscle fatigue observed during locomotion was reduced as indicated by the increase of MPF at the unaffected side (see Section 2) [4].

A previous human study indicated that effective gait training on treadmill with body weight support, improving walking speed and endurance, was characterized by an increase of brain activity in the bilateral primary sensorimotor cortices, the cingulate motor areas, the caudate nuclei bilaterally, and the thalamus of the affected hemisphere during paretic foot movement [84]. In addition, treadmill exercise (improving cardiovascular fitness by 18%) activates subcortical neural networks during single knee movements of the lower extremity, as observed by fMRI. Indeed, physical training changed brain activation during paretic limb movement, showing 72% and 18% increased activation in posterior cerebellar lobe and midbrain, respectively [85]. After 10 weeks of training, the improvement in sensorimotor function, assessed with the Fugl-Meyer Index, seemed strongly related to the improvement in aerobic capacity [86].

It is noteworthy that some neural mechanisms after physical exercise remain to be investigated such as diaschisis. Indeed, functional deficits may also be associated with distant effects after subcortical lesions resulting from deafferentation to a region not directly involved in the stroke. Moreover, it was found that several rehabilitation exercises, inducing blood flow and metabolic changes in the contralesional hemisphere, might act on diaschisis. Given that alleviation of diaschisis could contribute to motor recovery [87], it seems important that further studies will determine how endurance exercises may influence diaschisis after cerebral ischemia.

4. Influence of Exercise Parameters on Neuroplasticity and Functional Outcomes

Depending on the chosen exercise parameters (volume, intensity, session frequency, and timing of exercise initiation), neuroplasticity can be either adaptive or maladaptive to recovery and, thus, may affect the training effectiveness after cerebral ischemia [46, 87–91]. Strong evidence suggested a time-limited period of enhanced neuroplasticity [89, 92–94]. Animal studies underlined that the limb function was less improved when training started before 24 h after ischemia compared with a start during the 5 first days [89]. Moreover, early intense training might induce larger cortical infarct volume and thalamic atrophy when the program starts before 24 h [95]. Exercise detrimental effects were also observed on neuroplasticity when animals performed treadmill exercise shortly after trauma [96]. The lesional aggravation might be related to localized and prolonged hyperthermia. Indeed, physical exercise, known to induce hyperthermia,

could accentuate the cerebral ischemia-induced release of glutamate and catecholamines that lead to neural excitotoxicity [95, 97]. However, it seems important to add that other processes than hyperthermia might be involved in the sensorimotor deficit aggravations and need to be explored. Furthermore, early running training downregulated proteins involved in neuroplasticity such as MAPK, CAMKII, PKC, synapsin I, or CREB expression [98] and decreased the level of proinflammatory cytokines known to be related to neuroprotection [99, 100]. Finally, Yagita et al. (2006) had shown that two weeks of running exercise reduced the number of newborn neurons in ischemic rats and thus limited neurogenesis in the hippocampus [101]. The author suggested that running was too stressful and enhanced the corticosterone level, known to decrease neurogenesis. Likewise, an immediate overuse of the lesioned forelimb could also increase tissue loss around the lesion and aggravated sensorimotor deficits on a longer term [102–104]. One explanation could be related to anatomical damage resulting from the reduction of dendritic growth in the ischemic hemisphere [104].

In addition, an augmented volume of exercise or increase of inpatient therapy duration for stroke did not indicate superior effects on functional recovery and activities of daily living [105, 106]. Interestingly, when healthy active men were subjected to a strong increase of volume training, physical performance was also not improved [107]. It thus seems that volume is not the main exercise parameter that should be investigated for improving aerobic program.

Greater improvements were observed with higher exercise intensities after stroke and neurodegenerative disease but also for healthy individuals [92, 108–110]. However, the effect of intensity on neuroplasticity remains unclear because it is poorly investigated in stroke patients or in ischemic animal models (ongoing study in our laboratory). It should also be pointed that one major methodological limitation is related to the exercise intensity determination. Indeed, training intensity was mainly based on maximal oxygen consumption or maximal exercise heart rate (or even intensity based on empirical values). However, these parameters were not appropriated because stroke patients never reached maximal capabilities. It was recently suggested that prescribing intensity should rather be based on submaximal parameters such as ventilatory or lactic threshold that were more accurate in distinguishing moderate to intense exercise [111].

5. Beneficial Effect of Physical Training Rehabilitation in Combination with Pharmacological Treatments

Using multiple recovery processes (physical exercise and pharmacologic treatments) may be critical for enhancing functional outcomes, in contrast to monotherapies targeting single mechanisms (Table 1).

Given that recent findings have established that anti-inflammation may be an important target for stroke treatment [112], some authors demonstrated that combination of skilled reaching training with indomethacin or minocycline accentuated recovery [10]. First, beneficial effects of such combination included an improvement of sensorimotor function as indicated by higher correct placements of the impaired forelimb during a walking task. Secondly, the number of proliferating microglia was more reduced and the survival of newborn astrocytes in the peri-infarct zone was increased compared to rats that underwent training alone. Authors suggested that the motor outcome improvement might be partially explained by the observed changes of neural response.

In addition, therapeutic drug such as S-nitrosoglutathione (GSNO) exhibits similar neurovascular protecting effects compared to physical training following traumatic brain trauma and cerebral ischemia in rats [18, 27]. Administration of GSNO could reduce neuronal apoptotic cell death, excitotoxicity, and inflammation as well as protecting BBB integrity. GSNO could also stimulate the expression of VEGF and BDNF after traumatic brain injury. Several authors have demonstrated that combining rotating rod motor exercise with GSNO administration accelerated and accentuated both walking and balance abilities compared to the effects induced by each treatment applied separately. Moreover, the improved motor function was associated with a reduction of both infarct volume and neuronal cell death as well as an increase of PECAM-1 and BDNF expression [28].

D-amphetamine, acting primarily through norepinephrine and dopamine mechanisms, is a potent modulator of neurological function and cortical excitation that facilitated motor skill abilities [113]. It was demonstrated that a single injection of D-amphetamine on the first day of training facilitates effectiveness of motor skill training compared with D-amphetamine treatment alone after a focal cortical infarct in squirrel monkeys [29]. However, administration of a high dose of D-amphetamine combined with rehabilitation training failed to promote fine motor recovery in a rat embolic stroke model. Authors explained this result by the fact that the dose of D-amphetamine was too high, thereby limiting animal engagement in the staircase test [114].

Other pharmacological agents could improve motor recovery by focusing on different neural mechanisms. For example, Nogo-A protein, a myelin-associated inhibitor, appears to be partially responsible for inhibition of axonal growth in white matter. Therefore, suppressing this myelin-associated neurite outgrowth inhibitor by specific antagonists of Nogo-A, such as NEP 1-40 or NGR(310)Ecto-FC, increased neurite outgrowth and axonal regeneration after stroke [30, 31]. Five-week motor training combined with NEP 1-40 treatment accelerated motor recovery (skilled reach and foot fault tests) from the first week after cerebral ischemia compared to treatments applied alone in which beneficial effects were observed later (weeks 2 and 4) [30]. Unfortunately, neural plasticity mechanisms underlying this improvement remain unknown, but these findings showed that this therapy combination could accelerate the recovery process following cerebral ischemia.

Neurons and glial cells in brain can synthesize progesterone that exhibits neuroprotective effects after cerebral ischemia [115–118]. Rehabilitation could increase reorganization of cortical maps whereas the progesterone might reduce the infarct volume through its neuroprotective effect by

Table 1: Influence of pharmacological agents associated with physical exercise on motor recovery after brain stroke.

Drug agents	Targets	Results	References
Indomethacin Minocycline	Inflammatory processes	↘ infarct volume (indomethacin only) ↗ sensorimotor performance ↘ microglia ↗ astroglia	[10]
GSNO	Oxidative stress Inflammatory processes Excitotoxicity	↘ infarct volume, apoptotic cell death ↗ neurological score, motor recovery, and survival rate ↗ CBF, synaptic plasticity, and BBB leakage ↘ TNF-α, IL-1β, and iNOS	[18, 27, 28]
D-Amphetamine	Noradrenergic α1-receptor agonist	↗ motor recovery	[29]
NEP 1-40	Nogo-A protein inhibitor	↗ early motor recovery ↗ axonal growth	[30]
NgR(310)Ecto-Fc	Nogo-NgR pathway inhibitor	↗ motor recovery ↗ axonal plasticity	[31]
Progesterone	Excitotoxicity Inflammatory processes	↘ infarct volume ↗ forelimbs strength and motor recovery	[32]
EGF* and EPO*	Neuron proliferation, migration, and differentiation	↗ accelerated fine motor recovery	[33, 34]
Chondroitinase ABC	Chondroitin sulphate proteoglycans (CSPGs)	↘ CSPGs ↗ synaptic plasticity ↗ motor recovery	[35]

↗ indicates an increase and ↘ a decrease, respectively; NEP 1-40: NOGO extracellular peptide; EGF: epidermal growth factor; EPO: erythropoietin; GSNO: S-nitrosoglutathione; CBF: cerebral blood flow; BDNF: brain developed neurotrophic factor; trkB: tropomyosin receptor kinase B; BBB: blood brain barrier.
*Molecules combination.

targeting excitotoxicity. Although this combination had no additional effect in reducing infarct volume, functional recovery effectiveness was promoted as shown by the increased rotarod performance and forelimb grip strength at all time points within 7 days after ischemic stroke [32].

In addition, some authors indicated that combination of two pharmacological treatments could improve functional recovery and neural plasticity compared to either treatment alone. For example, association of epidermal growth factor (EGF) and erythropoietin (EPO) treatments is more effective in promoting tissue regeneration and proliferation of neural precursor cells than monotherapy [33]. However, an even greater improvement in skilled reaching ability in the staircase test was observed when physical rehabilitation was added to serial application of both EGF and EPO [34]. Authors have also indicated that fine motor skill improvement was observed 10 weeks after functional rehabilitation alone whereas 4-week-long treatment combination was sufficient to improve sensorimotor function. It means that functional recovery is also clearly accelerated with this combination.

Another strategy consists of recreating a tissue environment free from growth inhibiting molecules. Chondroitinase ABC, which degrades inhibitory chondroitin sulphate proteoglycans present in the extracellular matrix, reduces the neurite-inhibitory environment and facilitates axonal sprouting and sensorimotor recovery after spinal cord injury [119, 120] and after cerebral ischemia [121, 122]. This treatment was applied at the ipsilesional cortex in rats in combination with functional rehabilitation. Synaptic plasticity, assessed by measuring the expression of glutamate vesicular transporter (vGLUT1 and vGLUT2) and the GABA vesicular transporter (vGAT), and functional recovery were promoted by the synergic effect of these treatments [35].

Given that some promising treatments developed in preclinical studies failed to be effective in clinical studies [123, 124], it seems crucial to assess the pharmacological effectiveness in stroke patients before combining it with physical exercise. Therefore, we need to keep in mind that not all the effective combinations found in animal studies could be considered effective for human. Nevertheless, assessing both treatments and exercise in rodent models seems relevant to found new therapeutic strategies to highlight the most effective and applicable strategies for human.

6. Influence of Prestroke Physical Activity on Motor Dysfunction Severity

To avoid administration of several pharmacological substances in individuals with high risks of stroke (may induce deleterious interaction between drugs and complications due to their side effects), physical activity might be a major alternative preventive strategy for reducing the brain secondary injury when stroke occurs. Several recent human and animal studies have well demonstrated that preischemic physical activity may reduce initial stroke severity on functional motor outcomes, edema, and infarct volume by acting on inflammation, vascular processes, BDNF expression, and metabolic disorders [14, 16, 17, 125, 126].

Ischemia-induced brain inflammation is believed to play a pivotal role in the development of secondary brain injury by intensifying the inflammatory cell accumulation and microvascular impairments. Indeed, adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) promote leukocyte infiltration into injury site and leukocyte adhesion to microvascular endothelium. Preischemic physical training-induced neuroprotection was associated with a decrease in ICAM-1 mRNA expression and in the number of ICAM-1-positive vessels [17]. Consequently, leukocyte accumulation in damaged cortex and striatum vessels decreased, resulting in reduction of brain inflammation during reperfusion.

Brain angiogenesis is another neuroprotective mechanism for reducing the detrimental motor effect of cerebral ischemia. Physical training prior to cerebral ischemia increased short- and long-term effects on microvascular density and cerebral blood flow by promoting angiogenesis and increasing cerebral vasomotor reactivity. It was found that preischemic exercise improved vasorelaxation by enhancing VEGF levels into the ischemic region, which is known to activate eNOS and EPCs recruitment [8, 19]. Likewise, exercise decreased the endothelin-1 (a powerful vasoconstrictor agent) expression, thereby reducing the impact of vasoconstriction on cerebral blood flow [127].

Preischemic training could also attenuate brain damage by limiting metabolic disorders after cerebral ischemia. Indeed, 5′AMP-activated protein kinase (AMPK), phosphofructokinase-1 (PFK), and hypoxia-induced factor-1α (HIF-1α), involved in glycolysis [18, 128], were significantly higher in preischemic trained rats [129]. Moreover, the increase of glucose transporters in neurons and endothelial cells of the BBB (GLUT3 and GLUT1, resp.) results in an improved glucose oxidation immediately after cerebral ischemia, allowing a faster and more substantial increase in ATP production.

Moreover, it was postulated that preischemic exercise (30 min on a treadmill, 5 days/week for 3 weeks) decreases BBB dysfunctions and, thus, reduces infarct volume and edema as confirmed by the MMP-9 expression decrement [130, 131]. Preischemic treadmill training-induced neuroprotection in ischemic rats increased endogenous BDNF expression that leads to motor recovery improvement [16, 17, 67, 132]. Interestingly, the preischemic treadmill exercise improved the therapeutic effectiveness of postischemic treadmill training on motor function compared with animals performing it only after cerebral ischemia [53].

Neuronal excitotoxicity induced by excessive glutamate release is a major deleterious event of the brain secondary injury [133, 134]. It was found that preischemic treadmill training could also reduce the expression of glutamate receptors, mGluR5 and NR2B, reflecting a decrease of glutamate effect on surrounding cells [135–137]. Moreover, it was postulated that the reduction of infarct volume induced by 12 weeks of treadmill might be related to increase of NGF expression and this receptor, p75, known to promote neuroprotection against excitotoxicity and free-radical damage [138].

Individuals who were physically active prior to stroke have been shown to exhibit less deleterious functional outcomes, as indicated by higher Barthel Index scores as well as Oxford Handicap Scale [14, 15]. Indeed, it was recently revealed that patients with high levels of prestroke physical activity were associated with milder stroke severity at admission, with faster early motor improvement and with a lower final infarct volume [139]. Interestingly, it seems that simply walking 1 h/day during 5 days per week or doing a vigorous aerobic activity 1 h/day twice a week is sufficient to induce preventive effects. However, findings remain conflicting in human studies because no strong association between higher levels of physical activity and better functional outcomes after stroke was found in a large prospective cohort [126].

7. Conclusion

Although acute exercise appears to be useful for better understanding neural and motor recovery mechanisms, few studies have used this experimental model to investigate stroke physiopathology. Neural adaptations remain thus unclear. Furthermore, physical training appears to be promising to improve functional motor recovery by promoting neuroplasticity at different cellular and molecular levels. These beneficial effects seem accelerated and/or accentuated when exercise is combined with an additional pharmacological treatment. Nevertheless, optimal parameters of training and treatment need to be investigated to maximize/accelerate neuroplasticity and motor recovery and avoid undesirable effects of exercise. All these findings could help researchers and therapists to justify the effectiveness of their physical training programs in order to increase the patient willingness to regularly perform physical activity before and after stroke.

Conflict of Interests

Each author of the present review declared no conflict of interests.

Acknowledgment

The authors are grateful to Dr. Vincent Pertici for comments on earlier version of this paper.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 138979, 14 pages
http://dx.doi.org/10.1155/2015/138979

Review Article

Synaptic Plasticity and Neurological Disorders in Neurotropic Viral Infections

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Received 30 January 2015; Revised 16 June 2015; Accepted 18 June 2015

Academic Editor: Alexandre H. Kihara

Based on the type of cells or tissues they tend to harbor or attack, many of the viruses are characterized. But, in case of neurotropic viruses, it is not possible to classify them based on their tropism because many of them are not primarily neurotropic. While rabies and poliovirus are considered as strictly neurotropic, other neurotropic viruses involve nervous tissue only secondarily. Since the AIDS pandemic, the interest in neurotropic viral infections has become essential for all clinical neurologists. Although these neurotropic viruses are able to be harbored in or infect the nervous system, not all the neurotropic viruses have been reported to cause disrupted synaptic plasticity and impaired cognitive functions. In this review, we have discussed the neurotropic viruses, which play a major role in altered synaptic plasticity and neurological disorders.

1. Introduction

Over the years, Central Nervous System (CNS) has been shown to be the major target site for viral infections. Different viruses have different routes of entry and some viruses have been shown to penetrate the CNS (neuroinvasion) and can infect neurons and glial cells (neurotropism). Neurotropic viruses are categorized into neuroinvasive and neurovirulent groups and both of them are known to cause neuronal dysfunction. Interestingly, neuroinvasive virus is capable of accessing or entering the nervous system whereas neurovirulent virus is capable of causing disease within the nervous system. These neurotropic viruses such as coxsackie, Japanese, Venezuelan equine, and California encephalitis viruses, polio, mumps, echo, influenza, measles, and rabies cause acute infection. Other viruses that come under this category are members of the family Herpesviridae, such as Cytomegalo, Varicella-zoster, Herpes simplex, and Epstein-Barr viruses. The ones that cause a latent infection are Varicella-zoster and Herpes simplex viruses, whereas other viruses like measles, rubella, John Cunningham, and retroviruses such as human T-lymphotropic virus 1 and human immunodeficiency virus are also reported to be neuropathogenic [1]. All of these pathogens have different modes of entry into the human brain, causing the neuropathogenesis that leads to the neurocognitive disorders. However, the neuropathogenic mechanisms that are involved in these disorders neither are clear nor are elucidated yet and require further studies to identify the therapeutic targets. Neuropathogenic mechanisms that lead to these disorders need to be better understood to identify therapeutic targets.

Viral infections of the CNS that injure or destroy specific populations of brain cells are frequently associated with behavioral disturbances. These events occur either directly due to virus replication or indirectly as a result of the host immune response against the infectious agent. Neurotropic viruses can also persist in the CNS and, in the absence of cell destruction or inflammation, cause defects in goal-oriented behavior. Therefore, viruses may contribute to human CNS disorders whose etiology remains elusive. The finding of virally mediated impairment in neuronal function in the absence of cell destruction raises the possibility that noncytolytic viruses that persistently infect neurons may contribute to many human CNS disorders whose etiology

is unknown. Since neurons are not destroyed by the viral infection, antiviral therapies resulting in viral clearance from these cells may restore normal brain function. Studies to test this hypothesis are currently underway.

Borna disease virus (BDV) is an enveloped virus with a nonsegmented, negative-strand RNA genome belonging to the Bornaviridae family within the Mononegavirales order. This neurotropic virus infects a wide variety of mammals, and serological evidence suggests that BDV, or a BDV-like virus, also infects humans. Infected hosts develop a wide spectrum of neurological disorders, ranging from immune-mediated diseases to behavioral alterations without inflammation, reminiscent of symptoms observed in human psychiatric diseases such as schizophrenia, mood disorders, and autism [2, 3]. BDV has a noncytolytic strategy of replication and primarily infects neurons of the limbic system, notably the cortex and hippocampus [4]. To date, the mechanisms responsible for the cognitive impairment of BDV-infected animals are still poorly understood. It is possible that neuronal infection by BDV impairs signaling pathways that are important for proper neuronal functioning and neuronal communication. Recently, it was observed that BDV specifically interferes with the activity-dependent enhancement of synaptic vesicle recycling, one component of neuronal communication as well as synaptic transmission [5]. Accordingly, in this review, we have discussed synaptic plasticity changes and neurological disorders in neurotropic viral infections, which affects neurocognitive functions.

2. Synaptic Plasticity

Plasticity is fascinating and one of the most important characteristics of the mammalian brain. Synapses have the ability to undergo lasting morphological and biochemical changes according to different and specific types of neuromodulators and stimuli, which forms a cellular basis for memory and learning. However, the relationship between a specific type of memory and the form of its synaptic plasticity is still unclear [6]. The responses that are involved can cause neural activity to have the capacity to modify neural circuit functions, which will give as a result different thoughts, behaviors, and feelings. This modification affects the efficacy or strength of synaptic transmissions and for more than a century has been thought to play a critical role in the brain capacity to integrate temporary involvements/feelings into stable traces of memory. In addition, it has been thought that synaptic plasticity played an important role in neural circuitry development. Evidence has demonstrated that certain prominent neuropsychiatric disorders happened as a consequence of impairments in synaptic plasticity mechanisms. Overall, many synaptic plasticity functions as well as mechanisms and forms have been described. Changes in enhanced or suppressed synaptic transmissions can have a temporal span of milliseconds to days or even longer [7].

3. Short-Term Synaptic Plasticity

Nearly every synapse studied in a variety of organisms, from invertebrates to mammals, has shown various forms of short-term synaptic plasticity which lasts for few milliseconds to a couple of minutes [8]. It is believed that these forms of synaptic plasticity play a significant role in short-term adaptations to transient changes in behavioral states, short-lasting forms of memory, and sensory inputs. The majority of these forms are produced by short outbreaks of activity that come as a result of a temporary buildup of calcium in presynaptic nerve terminals. This increment in calcium causes modifications in the possibility of neurotransmitter release by changing the biochemical processes that causes the exocytosis of synaptic vesicles [7]. Short-term synaptic plasticity was initially recognized as behaviorally significant in studies of marine organisms such as *Aplysia* [9]. One of the main effects of short-term synaptic plasticity is to act on the information processing function of synapses, allowing them to perform as filters with different properties.

4. Long-Term Synaptic Plasticity

In the hippocampus, a repetitive stimulation of excitatory synapses is able to result in a potentiation of synaptic strength, lasting for hours to days, and it is referred to as long-term potentiation (LTP) or long-term synaptic plasticity. Different forms of long-term depression (LTD) are present in the majority of synapses that show LTP. LTD is an activity-dependent decline in the efficiency of neuronal synapses, resulting in a long patterned stimulus. Therefore, an important idea is that different patterns of activity are able to modify synaptic strength in a bidirectional way at excitatory synapses. Homeostatic plasticity has been recently recognized as an additional form of synaptic plasticity [10] as well as metaplasticity [11]. Schematic representation of the synapse (Figure 1(a)), establishing LTP (Figure 1(b)), and synapse exhibiting LTP (Figure 1(c)) was shown in Figure 1. Figure 2 is showing the different mechanisms of long-term depression.

5. Rabies Virus

Rabies virus (RV) belongs to the Rhabdoviridae family and infects many animals (bats, skunks, foxes, and dogs) and human beings. RV in animal resides in salivary glands and spreads among different hosts via bites/scratches. RV infected animals can survive for years secreting virus particles in their saliva. In contrast to other infected animals, human infection results in fatal acute myeloencephalitis in untreated patients. By binding to acetylcholine receptors (nAchR) and neural cell adhesion molecules (NCAM), RV enters the axons of motor neurons at the neuromuscular junction [12]. Transneuronal spread occurs exclusively between synaptically connected neurons and the infection moves unidirectionally from postsynaptic to presynaptic neurons (retrograde spread). Once rabies infection reaches the CNS, marked behavioral and neurological symptoms begin and death almost always ensues [13]. In contrast to neuronal dysfunction related severe clinical manifestations, in postmortem examinations, only mild lesions in the CNS were observed. Various studies reported that fetal rabies causes neuronal dysfunction, including ion channel dysfunction and neurotransmitter abnormalities rather than neuronal damage [14–16], and downregulation

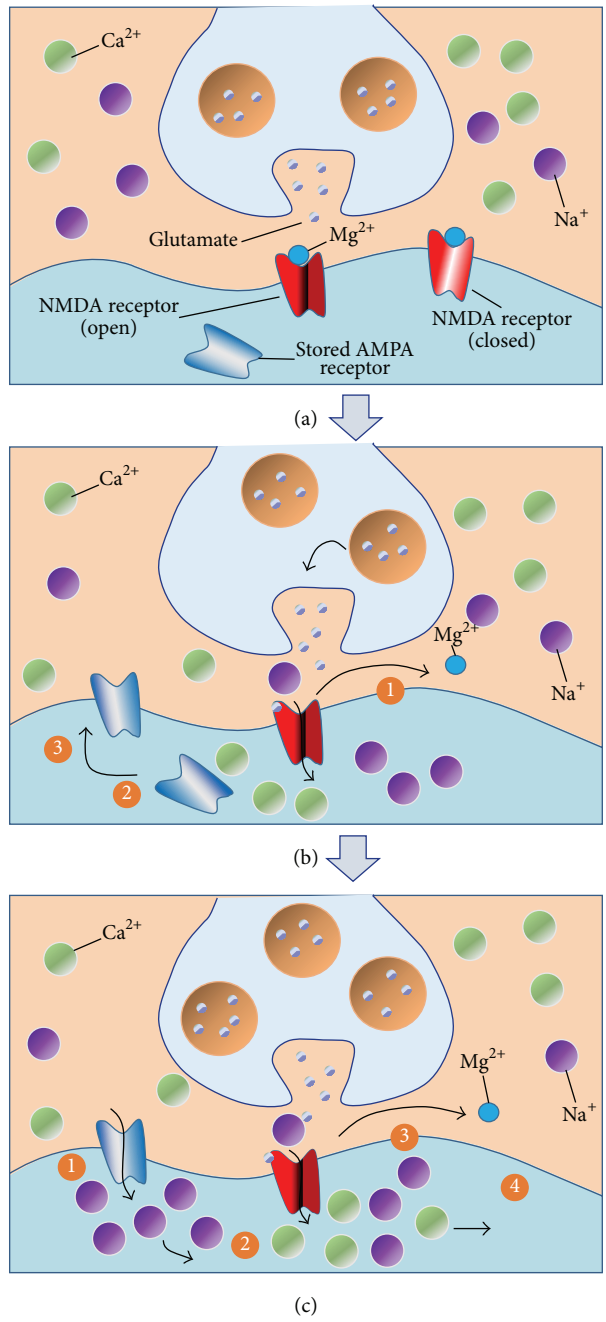

Figure 1: (a) Synapse prior to long term potentiation: NMDA and AMPA are two types of receptors at the postsynaptic neuron, for the neurotransmitter glutamate. NMDA receptors open in response to glutamate prior to potentiation. However, they are blocked by Mg^{2+}. (b) Establishing LTP: NMDA receptors release Mg^{2+} after depolarization of the postsynaptic membrane in response to the activity. Na^{+} and Ca^{+} travel inside and induce the migration of internal AMPA receptors to the membrane. (c) Synapse exhibiting LTP: NMDA receptors are unblocked when depolarization is triggered by AMPA receptors. These two receptors are now responsible for action potentials.

of synaptic plasticity regulated protein has been reported in the silver haired bat rabies virus infection. Downregulation of these synaptic plasticity proteins leads to the blocked synaptic vesicle recycling, therefore, the reduced release and uptake of neurotransmitters [17]. Song et al. reported the decreased spine density in the street rabies virus infected hippocampus of mice and also reported that these changes were related to the depolymerization of filamentous actin (F-actin), a cytoskeleton protein that helps to regulate the morphogenesis and dynamics of dendritic spines [18].

6. Poliovirus

Poliovirus is part of the Picornaviridae family and enterovirus subgroup. The virus enters into the host and starts multiplying in the place of implantation, which is usually

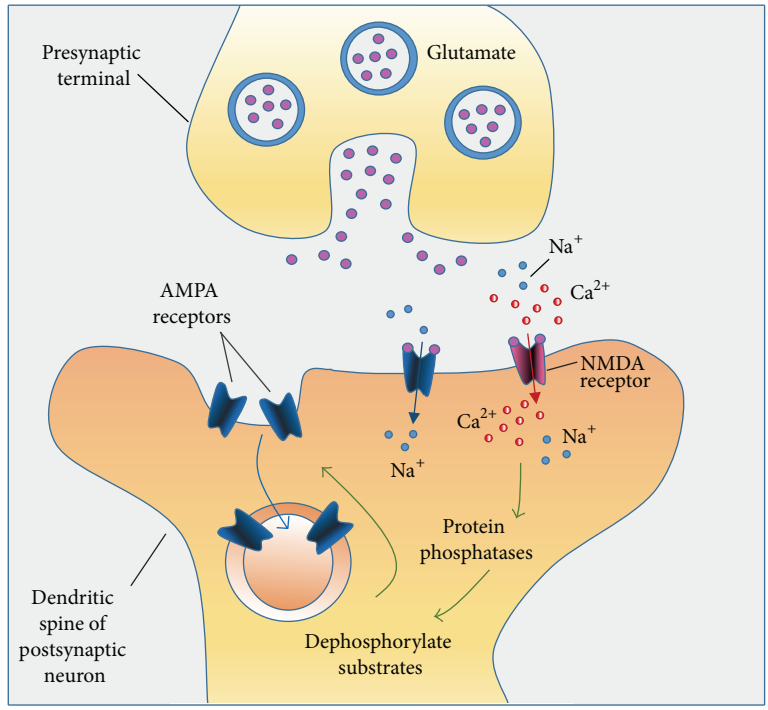

Figure 2: LTD mechanism: (a) Ca^{2+} ions enter in small quantities through NMDA receptors. (b) Activation of protein phosphatases. (c) Dephosphorylation of AMPA receptors leads to endocytosis of AMPA.

the gastrointestinal tract and pharynx. Before the onset of illness, the virus is present in the stool and throat. After the onset of the disease, the virus is less in the throat, but it will still be present in the stool for few weeks. Poliovirus is also capable of entering the bloodstream, invading lymphoid tissue, and subsequently infecting the CNS. Poliovirus was reported to enter the neurons by a receptor-mediated endocytosis at the neuromuscular junction and traverse from nerve terminal to the cell body using the host retrograde axonal transport system. It can hijack the host transport machinery using Tctex-1, a component of the dynein light chain involved in retrograde axonal transport [19, 20]. In *in vivo* intramuscularly injected mice with poliovirus, it was observed that trafficking of poliovirus in peripheral nervous system is difficult due to inefficient retrograde axonal transport and this could be the reason for low incidence of paralytic poliomyelitis in humans [21]. It replicates in motor neurons of the brain stem and the anterior horn, which leads to poliomyelitis and cell destruction. About 95% of the infected patients are asymptomatic and nearly 4%–8% of cases show a nonspecific but minor disease that does not invade the CNS. In few days after a prodrome, nonparalytic aseptic meningitis happens in 2% of infected patients, whereas flaccid paralysis happens in less than 1% of infections. After prodromal symptoms, paralytic symptoms begin and these symptoms usually progress for the next 3 days. Usually, when the temperature goes back to standard levels, there is no additional paralysis.

Of all poliomyelitis survivors, only 25% showed a relapse of fatigue, weakening of the muscles, or paralytic symptoms, and this is referred to as postpolio syndrome. It has been suggested that the cause of this syndrome is a reappearance of latent virus. However, there is no evidence yet to support this argument [22]. After the initial acute infection, there are some recovery mechanisms that occur in a short period of time. Patients can experience temporary paralysis, followed by a partial paralysis recovery. This temporary paralysis could be happening as a consequence of temporary silencing of neurons by transient virus infection, neuronal response to transient inflammation or to transient release of inhibitory neuroactive agents. Another probability would be when local plasticity allows replacement of lost motor neurons by cytolytic virus infection with uninfected neurons. The mechanisms behind the loss of motor neurons are still unclear. An understanding of this mechanism may give us an insight of postpolio syndrome as well as other motor neuron illnesses [23].

Some data found in poliomyelitis cases demonstrated that redundant systems and cells take over when loss of neurons occurs. This is one of the main characteristics of neurological diseases. However, there is a certain limit to the amount of neurons that can be lost. Therefore, after a certain percentage, symptoms only start to be more evident. For instance, when dopamine neurons are lost in Parkinson's disease, it leads to muscle rigidity and tremor instead of the flaccid paralysis seen in PV. Narcolepsy is another example, in which hypocretin neurons are lost. This loss results in a temporary flaccid paralysis during cataplexy. Positive correlation has been observed between the severity of symptoms and the number of neurons lost. However, asymptomatic cases

are seen if there is less than 50% loss of neurons. In this perspective, the combination of initial killed cells by PV and the loss of neurons as a consequence of aging may lead to the manifestation of postpolio syndrome. As a result, there would be a high probability of insufficient motor neurons to continue with normal functions on the affected muscles. An alternative could be to target motor neurons with a low-grade autoimmune mechanism when they are PV-initiated [23].

7. Japanese Encephalitis

Japanese encephalitis (JE) is caused by the JE virus (JEV), which is a positive-sense and single-stranded RNA virus that forms part of the Flaviviridae family. The transmission of this virus occurs in a zoonotic way between water birds, pigs, and mosquitoes. Humans are a dead end host because of low level and transient viremia after being infected accidentally [24]. This disease is most commonly seen in Southeast Asia, where it affects about 50,000 individuals and causes approximately 10,000 deaths per year. In new endemic areas, both children and adults are found to be affected and, on the other hand, mostly children are affected in regions where this infection has been endemic for several years. In few areas, such as Korea and Japan, this virus has been controlled for a long time by immunization. In these areas, the virus may affect the elderly only. Case studies reported that children with JE have severe encephalitis characterized by a high frequency of seizures, deep coma, and mortality rates. In addition, seizures are most commonly found in 64% to 80% of children in comparison to only in 10% of adults [25–27].

The possible route of CNS entry of JEV is through the capillary endothelial cells (CEC), as the entry between CECs is inhibited by tight junctions [28]. In an *in vivo* mouse model of intravenous JEV infection, it was shown that viral titers increased exponentially in the brain (propagates in neurons) 2–5 days after infection that led to the exponential increase in the inflammatory cytokines and chemokines in the brain. Increased blood-brain barrier (BBB) permeability was observed only after 4th day of postinfection [29]. Most individuals that survived JEV infection experience severe neurological sequelae, such as language and cognitive impairments, motor deficits, and learning difficulties. In JE individuals, neuronal death can be caused by either the virus or as a bystander method facilitated by a strong inflammatory attack and microglial activation [30, 31]. Neuronal loss is regulated by the CNS by inducing the differentiation of new astrocytes and neurons from inhabitant multipotential neural progenitors cells (NPCs) [32]. These NPCs have the ability to self-renew over their lifespan and are located in neurogenic zones such as the dentate gyrus of the hippocampus and the subventricular zone (SVZ) [33]. Active NPCs are vastly lost from the SVZ by inhibiting their cycling ability as a result of JEV infection. Therefore, the formation of neurospheres by SVZ cells is greatly affected when they are JEV infected. The critical postnatal age is a predominant target and decreases the NPCs population in the SVZ and damages the recovery process. These might have a critical effect in JE survivors and their neurological outcomes [34]. JEV-infected microglia secretes inflammatory molecules that cause death of bystander neurons. Certain proinflammatory cytokines such as IL-6, TNF-α, and ROS/NO and MCP-1 are secreted in high concentrations by the infected microglia [35]. Secretions in high levels of these factors are antineurogenic and neurotoxic [36, 37].

8. Influenza Virus

Influenza is a serious health concern and economic burden since it remains as the primary cause of disease and death worldwide. Even though a lot of individuals recover from this infection, the short- and long-term effects on the CNS remain unclear. Cognitive and neurological consequences related with this virus have been described for many decades after the 1918 "Spanish" flu, as well as during the pandemic of influenza A H1N1. However, mechanisms associated with the symptoms are still unclear [38–41]. Most influenza strains are nonneurotropic, including the ones responsible for pandemics [42–44]. This suggests that neurological symptoms do not happen as a result of direct CNS viral infection but because of a neuroinflammation that came from an induced peripheral viral infection.

The peripheral innate immune system has been reported to get activated, producing certain cytokines such as interleukin-1β (IL-1β), IL-6, and TNF-α within the brain. As a result, this activation can have deleterious effects on emotional and cognitive behavior [45–48]. Long-term potentiation can be directly impaired and neurotrophins inhibited [49] by inflammatory cytokines [50, 51]. Neurotrophins are important for memory formation, synaptic plasticity, and neuronal function and survival [52–54]. Also, hippocampal neuronal morphology alterations occur after central and peripheral administration of lipopolysaccharide (LPS) takes place, inducing an innate immune response [55, 56]. While spine density and dendritic branching changes have an effect on synaptic plasticity [57, 58], induced inflammation alterations in neuronal complexity result in a hippocampal function deficit related to memory and learning. Infected mice with influenza A/PR8/34 (H1N1) were observed to have cognitive deficits and hippocampal neuroinflammation that were related to substantial changes in dentate gyrus neuron morphology and CA1 as well as the loss of neurotrophic factors [59].

9. Herpes Simplex

Herpes simplex virus (HSV) is a double stranded DNA virus. It was shown to enter the brain amygdala and hippocampus through the olfactory nerve and locus coeruleus. It has the tendency to enter latency within the CNS. In the infected mice model, both primary infection and reactivation of latent DNA in the brain led to neuronal damage that resulted in loss of memory, learning deficits, and behavioral change [60, 61]. In addition, it is transported transsynaptically, anterogradely, and retrogradely. HSV infection of the CNS can be lethal by affecting the inferior and medial temporal lobe. Some of the symptoms seen in acute Herpes simplex encephalitis

are Wernicke's aphasia, headache, fever, epileptic seizures, confusion, and low consciousness. Memory impairment may persist when the limbic system and temporal lobe are affected [62].

The viral DNA was found in very few young people and children's brains in comparison to elderly brains [63, 64], which may prove that HSV1 enters into older people's brain as a result of a weakened immune system. In addition, 60% of patients who are carriers of the APOE-e4 allele show a higher risk factor for Alzheimer's disease (AD) when the virus is present [65]. It has also been reported that HSV1 can be reactivated in brain, producing a recurrent infection [63]. Most of the time, HSV1 results in cell death. As a result, it was suggested that HSV1 might be reactivated during stress, inflammation, or immunosuppression conditions which may lead to the neuronal damage and subsequently to the development of AD, especially in APOE-e4 carriers. Neuropathological processes in case of HSV1 acting with APOE-e4 might occur due to the accumulation of AD-like tau (P-tau) and beta amyloid (Aβ) [63, 65]. Reactivation events are known to occur in the peripheral nervous system. HSV1 is located in the trigeminal ganglia, where it causes an evident damage by the appearance of cold sores in approximately 40% of infected individuals.

10. Varicella-Zoster

Varicella-zoster virus (VZV) is a human alphaherpesvirus that infects up to 90% of the human population. Following primary infection (varicella or chicken pox) which is more common during childhood, the virus establishes a lifelong latent infection in the dorsal root ganglia of the host and it may cause neurological complications such as postherpetic neuralgia (PHN), zoster-associated pain (ZAP), encephalitis, segmental motor weakness, myelitis, or arteritis, which may be fatal or may be followed by significant morbidity [66–70]. The main clinical characteristics of Herpes-zoster are dermatomal rash, acute pain, and neurologic symptoms [71]. Encephalitis and meningitis have also been observed to be caused by VZV [72]. The CNS complications can occur during primary infection and in the reactivation of VZV. The more serious complications occur when VZV invades the spinal cord or cerebral arteries after reactivation of the virus. The most common complication in 7 to 35% of infected individuals is PHN. Its symptoms involve constant, severe, stabbing or burning, dysesthetic pain. Although pathogenic mechanisms of PHN are unknown, two possible mechanisms are altered excitability of ganglionic or spinal cord neurons and persistent or low-grade productive virus infection in ganglia [73–75]. It has been observed that primary VZV infection causes VZV to be persistent in dorsal root and cranial nerve ganglia [71, 76–78]. When reactivation of VZV occurs, the feature dermatomal rash of Herpes-zoster takes place due to the movement of VZ virions through neuronal cell bodies into the skin. Weakness or paralysis of ipsilateral facial muscles is caused due to the zoster infection of the seventh cranial nerve (geniculate) ganglion [79]. Lower motor neuron type weakness in the arm and leg is caused by the cervical or lumbar distribution of zoster, respectively [80, 81].

11. Cytomegalovirus

Cytomegalovirus (CMV) is a common intrauterine pathogen that causes congenital developmental abnormalities of the CNS and developmental neurological disabilities such as CMV encephalitis, characterized by focal areas of reactive gliosis, reactive mononuclear cells, microglial nodules, and ventriculoencephalitis [82]. In the immunocompromised patients, CMV was reported to reach the brain from the blood and disseminated further by the CSF prior to the subsequent movement into the brain parenchyma [83]. It has been observed to be a lethal ventriculoencephalitis in individuals with advanced AIDS [84]. It has also been reported that CMV infects more cells in the subventricular and ventricular areas of the brain in congenital infected adults and children [84–86]. These results have also been observed in congenital CMV mouse models [87]. Impairment of neural stem cells (NSCs) may result in neuropathological effects that are related to CMV brain infection [88]. Currently, the leading cause of childhood disorders as well as birth defects in the United States is the congenital CMV infection. Every year, about 8,000 children show some neurological sequelae that are associated with congenital CMV infection. Nevertheless, the neuropathogenesis of this infection is still unclear. Human neural precursor cells have been reported to be vulnerable to CMV infection [89–91]. Alteration of the cellular differentiation process of these cells is observed in the presence of CMV infection [91, 92]. In CMV infected mice models, the expression of immediate early (IE) genes was held in the postnatal infected brain cortex. This might have happened because of the development of infected NSCs [93]. Likewise, IE expression in the cerebellum is related to the late development and movement of precursor cells [94]. A better understanding of the relationship between NSCs and CMV is critical for the development of neuropathogenic mechanisms of viral infection.

12. Epstein-Barr Virus

Epstein-Barr virus (EBV) is a human herpesvirus related to epithelial and lymphoid malignancies. This virus causes transmissible mononucleosis. Occasionally, EBV is said to produce an extensive variety of CNS infections, such as Guillain-Barre syndrome, Bell Palsy, transverse myelitis, cerebellitis, aseptic meningitis, and encephalitis [95–98]. These neurological complications occur during primary infection, typically in childhood. For the first time, role of EBV in the development of multiple sclerosis was reported by Fraser et al. [99]. Multiple sclerosis is a chronic demyelinating disease of the CNS causing axonal pathology and episodic or progressive neurological disability [100]. The role of EBV in the pathogenesis of multiple sclerosis could be due to the molecular mimicry between EBV and CNS antigens that results in immunological cross-reaction and resultant autoimmune damage in the CNS [101]. There is a strong correlation between the frequency of CD8+ T cells and EBV

infected B cells in the CNS and it indicates that immunological response found in the multiple sclerosis is primarily against EBV, with bystander damage to the CNS [102, 103].

13. Human T-Lymphotropic Virus 1

Human T cell leukemia virus type 1 (HTLV-1) is a type C retrovirus. Even though most of infected patients do not show any symptoms, HTLV-1 is responsible for adult T cell leukemia (ATL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), which is a progressive demyelinating disorder [104]. HAM/TSP is a progressive and chronic inflammatory illness. In HAM/TSP, a deprivation of white matter can be observed inside the lateral funiculi spinal cord in the lumbar and thoracic tissue segments. The brainstem and cervical spinal cord have also shown degeneration, even though this might have happened as a result of Wallerian degeneration [105, 106]. The primary region of neuronal damage was found inside the corticospinal tract. Patients who exhibited this damage reported weakness in their lower limbs [107]. HAM/TSP is usually present as a spastic paraparesis. Sexual and urinary dysfunctions as well as lower back pain are some of the common symptoms [104, 108, 109]. HAM/TSP can be divided into two phases. The first phase is seen as an inflammatory response and the second phase as a chronic degenerative stage [110]. The inflammation seen in the first phase affects the BBB and lymphocyte trading into the CNS increases its chances of happening [111–114].

CNS cell loss and demyelination, occurring in HAM/TSP individuals, could involve different mechanisms. Some of these are the autoimmune mechanism of molecular mimicry, the direct damage mechanism, and the bystander mechanism [115]. HTLV-1-associated pathogenesis inside the CNS could be related to an autoimmune mechanism that includes molecular mimicry. In addition, the direct damage mechanism involves the infiltration of activated CD8+ cytotoxic T lymphocyte cells specific for HTLV-1 Tax protein. This indicates a continuous manifestation of viral proteins or replicating virus [116]. In this case, cellular damage occurs from the release of inflammatory molecules and the directed lysis of infected cells. The bystander mechanism involves the release of proinflammatory cytokines in response to HTLV-1, which causes damage in the CNS [117]. Proinflammatory cytokines such as interferon-γ (IFN-γ) and TNF-α are proposed to cause loss and dysfunction of CNS cells as well as disruption of the BBB [118].

Even though neurons are not proposed to harbor virus *in vivo* [119], HTLV-1 neuronal infection demonstrated the potential to do so *in vitro* by neuroblastoma cell line infection and nontumorigenic origin neuronal cell line, such as HCN-1a and HFGC [106]. As mentioned above, another proposed mechanism is the autoimmune pathology of molecular mimicry. This mechanism involves the recognition of a host antigen as a viral protein by the immune system.

14. Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a neurotropic virus that goes into the brain briefly after the infection [120]. HIV causes neurotoxic and inflammatory host responses by replicating in brain microglia and macrophages. HIV infection can also lead to neurological disorders known as HIV-associated neurocognitive disorders (HAND). Motor, behavioral, and cognitive abnormalities can be observed in HAND. HIV-1 is classified into three groups (M, O, and N) and into nine genetic subtypes (A–K). Among these, clades B and C are the most circulating HIV-1 variants (>86%) [121] worldwide. In North America, Australia, and Western Europe, the leading one is clade B, whereas, in Latin America, Africa, and Asia, the most common one is clade C. Before the use of highly active antiretroviral therapy (HAART), 30% of advanced HIV-1 infected individuals showed HIV-associated dementia (HAD) symptoms [122, 123]. On the other hand, Satishchandra et al. (2000) [124] along with other studies [125] stated a very low frequency of HAD in about 2% of patients that were HIV-1 clade C infected from India. After the introduction and use of HAART worldwide, the frequency of HAD has reduced significantly. However, 40–50% of patients still show symptoms related to HAND [126–130]. HIV is transported by infected perivascular macrophages and monocytes through the BBB. A decreased neuronal function and plasticity were observed in postmortem brains of HAND patients. These can be seen at systemic and cellular levels. At the cellular level, HAND patients showed a decreased dendritic and synaptic density as well as a synaptodendritic damage [130], which can cause a neural network interruption and eventually lead to caspase-3-dependent neuronal apoptosis [131]. This can be observed at the system level as white and grey matter degeneration in cortical and subcortical areas [120, 132]. The basal ganglia are mainly affected [133, 134]. Recently, we have reported dysregulated synaptic plasticity genes expression in clade B infected SK-N-MC neuronal cells and clades B and C infected astrocytes. We have observed induced apoptosis and decreased spine density in clade B infected neuronal cells compared to clade C infected and control cells. These observations indicate that HIV-1 clade B is more neuropathogenic than clade C [135]. In the process of exploring the epigenetic regulation of synaptic plasticity genes expression in HIV infected neuronal cells, we have observed HDAC2 upregulation in these cells. Inhibition of HDAC2 by using the vorinostat resulted in the recovery of synaptic plasticity genes expression in HIV infected neuronal cells [136]. In HIV infection, the leading cause of reduced neuronal function may be due to the synaptodendritic injury rather than neuronal loss. Furthermore, a difficult issue for neuro-AIDS is the number of HIV-positive individuals that abuse illicit drugs. Heroin abuse is a major risk factor for HIV transmission, while abuse of stimulants has become one of the risk factors for HIV. Alcohol and other drugs of abuse cause oxidative stress to increase as well as brain atrophy and bad performance in neurocognitive assessments [137].

15. HIV Induced Neuroinflammation and Neurotoxicity

A better understanding of the cellular and molecular mechanisms of HIV neurotoxicity is required for the prevention

of HIV neuropathology. HAND individuals usually experience prolonged symptoms of HIV encephalitis. In this neuroinflammatory condition, the presence of HIV-infected microglial cells, multinucleated giant cells, myelin loss, development of microglial nodules, and astrogliosis is observed [138–140]. When microglia, macrophages, and distressed astrocytes get activated, the uptake of excitotoxic neurotransmitters is reduced, inhibiting plasticity [141]. As a result, the formation of dendritic synapses and spines is also reduced. Furthermore, neuronal survival is compromised when the release of IL-1β, TNF-α [142], and CXCL12 [143] by infected glial cells takes place [144–146]. Therefore, glial cells have the capacity to decrease homeostasis-mediated plasticity by promoting or exacerbating HIV-induced neurotoxicity. *In vitro* data have demonstrated that cytokines can encourage neuronal loss. Nevertheless, microglia's role in HIV neuropathology is still unclear, as microglia can also be activated by dying and distressed neurons. Additionally, the basal ganglia exhibit a selective susceptibility to synaptodendritic injury that cannot be described only by inflammatory cytokines. All these data support the idea that HIV stimulates the release of different viral proteins and soluble host cell-derived factors that may collaborate to cause the pathology of synapses.

16. Role of HIV Proteins in Neurotoxicity

Out of nine HIV proteins reported to cause neuronal injury, the transactivator of transcription (Tat) protein is one of the major viral proteins that is able to cause neurotoxicity. Tat is vital for HIV replication and influences transcription initiation and elongation [147] at the HIV promoter. In addition, Tat can reduce neuronal survival by different mechanisms, such as inflammatory cytokine [148], impairment of mitochondrial function [149], and activation of ionotropic glutamate receptors [150]. HIV-infected cells can release Tat [151] and it has been observed that the combination of HIV-1 clade B and Tat protein intensifies the production of reactive oxygen species and inhibits redox expression compared to clade C or its Tat protein. These data show that HIV-1 clades B and C produce different effects of thiol alteration and redox expression. In addition, HIV-1 clade B induces oxidative stress, which leads to more immunoneuropathogenesis than HIV-1 clade C [152]. Recently, we have reported that clade B Tat differentially regulates the synaptic plasticity genes expression compared to clade C [153]. While penetration of antiretroviral drugs across the blood-brain barrier might be crucial for the treatment of HAND, we are using the nanotechnology based approach to inhibit HIV infection and latency in the CNS cells by transferring the anti-HIV drugs coupled with vorinostat [154]. Nef, Vif, Vpr, and Vpu are key accessory proteins in HIV pathogenesis that affect some host cell functions, such as cytoskeleton contraction [155], and promote the release of virions and optimize viral replication [156]. Once these proteins are released, they can induce neuronal apoptosis [157] throughout different mechanisms, such as the activation of caspase-8 (Vpr) and formation (Vpr and Vpu) or direct binding (Nef) to ion channels. This will lead to lethal abnormal membrane depolarization [158].

Glycoprotein gp120 is another structural protein that has been reported to induce neuronal apoptosis. This gp120 has a significant function in the viral infection cycle and binds to chemokine coreceptors CCR5 and CXCR4, allowing conformational change and entering of the virus into cells [159]. Neuronal apoptosis can be induced by a short exposure of neurons to gp120 [160, 161]. It has also been reported to stimulate axonal degeneration [162] as well as dendritic injury [163, 164]. These two main effects are associated with the synaptodendritic atrophy seen in HAD [165]. Gp120 transgenic mice have been reported to show dendritic reduction and neuronal loss [166], which indicates that gp120 is capable of reducing and affecting synaptic plasticity. Figure 3 is showing the role of HIV infection, Tat, and gp120 in the HIV-induced neurotoxicity.

In neuronal diseases including neurotropic viral infections, the peripheral innate immune system has been reported to get activated, producing certain cytokines within the brain. As a result, this stimulation can have deleterious effects on synaptic plasticity, emotional, and cognitive behavior. While current research in this area is ongoing, the role of synaptic plasticity during neurotropic viral infections and associated neurodegenerative diseases are the most recent and least understood. While there is an agreement that many neurodegenerative diseases are characteristic of a vigorous inflammatory response, it remains unclear how this process is related to disease processes. The interaction of viruses with their hosts is remarkable in numerous ways. The interaction between virus and host *in vivo*, especially in brain, is very complicated by the categorized arrangement of cells, tissues, and systems, which offer the appropriate protective response. If this united response to viral infection is not sufficient, then there is every possibility of resulting in disorders associated with synaptic plasticity and cognitive effects. Damage to brain cells can result from viral replication or by the action of the activated immune system and may result in the death of neuronal cells. Designing methods such as live-cell and intravital imaging together with neuron culturing methods supplemented by the capability to construct recombinant viruses will enable researchers to study some of the fundamental characteristics of virus replication as well as spread within and between neurons. These methodologies will empower the scientists to understand the mechanisms of how neurotropic viruses get entrance to and spread in the brain. New methods, such as deep sequencing of viral nucleic acid from clinical samples or single-molecule sequencing will enable identification of more neurovirulent and/or neuroinvasive virus mutants and will provide a genetic view of host barriers and viral bypass mechanisms and will help in improving the other cognitive associated neurodegenerative disorders.

Conflict of Interests

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

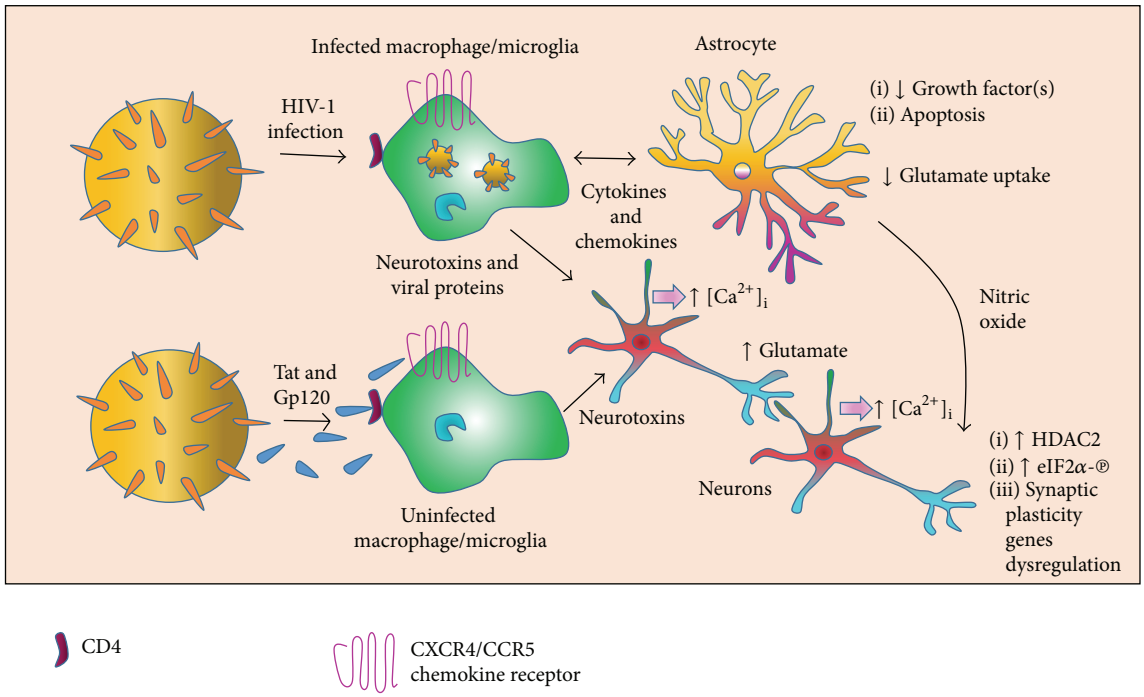

Figure 3: A model of HIV-induced neurotoxicity. Infected microglia or macrophages release viral proteins, chemokines, and cytokines. This activates uninfected microglia and macrophages. Neuronal injury, synapse damage, and cell death occur because immune activated and HIV-infected brain microglia and macrophages release neurotoxic elements. Excessive influx of Ca^{2+} ions occurs because of the overactivation of NMDA receptor-coupled ion channels that mediate neuronal injury. As a consequence, potentially harmful enzymes, release of glutamate, and free-radical formation are triggered. Subsequently, glutamate overstimulates NMDA receptors on nearby neurons, which causes additional injury. Upregulation of HDAC2 expression and increased eIF2α-phosphorylation leads to the dysregulated synaptic plasticity gene and protein expression which results in impaired synaptic plasticity.

Acknowledgment

This work was supported by National Institute of Health Grants 1R01DA027049, 1R01MH085259, and R01DA034547.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 798481, 13 pages
http://dx.doi.org/10.1155/2015/798481

Research Article

Brain Plasticity following Intensive Bimanual Therapy in Children with Hemiparesis: Preliminary Evidence

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Received 30 January 2015; Revised 10 June 2015; Accepted 11 June 2015

Academic Editor: Lin Xu

Neuroplasticity studies examining children with hemiparesis (CH) have focused predominantly on unilateral interventions. CH also have bimanual coordination impairments with bimanual interventions showing benefits. We explored neuroplasticity following hand-arm bimanual intensive therapy (HABIT) of 60 hours in twelve CH (6 females, mean age 11 ± 3.6 y). Serial behavioral evaluations and MR imaging including diffusion tensor (DTI) and functional (fMRI) imaging were performed before, immediately after, and at 6-week follow-up. Manual skills were assessed repeatedly with the Assisting Hand Assessment, Children's Hand Experience Questionnaire, and Jebsen-Taylor Test of Hand Function. Beta values, indicating the level of activation, and lateralization index (LI), indicating the pattern of brain activation, were computed from fMRI. White matter integrity of major fibers was assessed using DTI. 11/12 children showed improvement after intervention in at least one measure, with 8/12 improving on two or more tests. Changes were retained in 6/8 children at follow-up. Beta activation in the affected hemisphere increased at follow-up, and LI increased both after intervention and at follow-up. Correlations between LI and motor function emerged after intervention. Increased white matter integrity was detected in the corpus callosum and corticospinal tract after intervention in about half of the participants. Results provide first evidence for neuroplasticity changes following bimanual intervention in CH.

1. Introduction

Cerebral palsy (CP) results from early brain injury, either pre- or perinatal, and affects 2-3 in 1000 children. Approximately 30% of children with CP have hemiparesis, which manifests as motor impairments and weakness on one side of the body and causes substantial functional impairment in day-to-day tasks [1]. Beyond unilateral impairments, children with hemiparesis (CH) also have impairments in bimanual coordination [2].

Several types of intervention have shown success in improving hand function in hemiplegia, the most common of which are constraint induced movement therapy (CIMT), which involves unimanual training [3]. Another less studied therapy is hand-arm bimanual intensive therapy (HABIT) [4, 5], which involves practice of tasks requiring two hands in order to develop use of the affected hand and improve coordination. This type of bilateral intervention has demonstrated substantial benefits in this population [6].

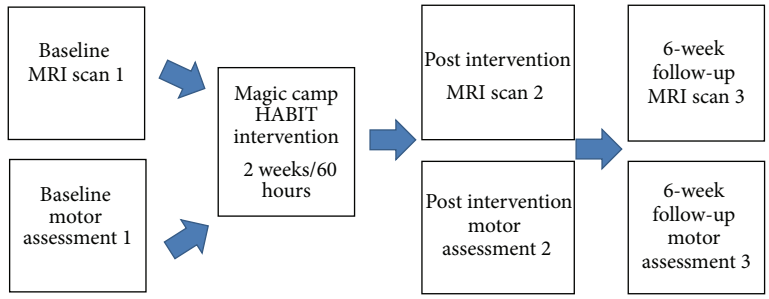

Figure 1: General study set-up for Magic camp HABIT intervention.

Advanced MRI methods, including functional magnetic resonance imaging (fMRI) and diffusion tensor imaging (DTI), have been shown to provide important information in the evaluation of CP and therapy response assessment [7]. fMRI studies have shown abnormal patterns of activation with a shift towards bilateral activation in CH [8]. Reduced white matter (WM) integrity in corticospinal tracts in the affected hemisphere was also shown using various diffusivity indices, corresponding to severity of hand function impairment [9].

However, little is known about changes in brain structure and function following motor interventions in CH since few studies have used serial imaging. A recent systematic review of the literature found just seven studies, all with small samples (3–10 children) [10]. While research into brain plasticity following rehabilitative interventions in stroke patients is more abundant [11–13], the later time of injury prevents comparison with unilateral CP, which commonly results from pre- or perinatal injury.

The existing studies describing neuroplastic changes following treatment involved either virtual reality intervention [14] or most often unimanual interventions, such as CIMT [10, 15, 16]. A review of the literature reported enlargement of the primary hand motor area (M1) in the affected hemisphere following intervention, with no consistent effects in the less affected hemisphere [10]. A previous study reported change in LI towards unilateral pattern associated with greater improvement in motor function after CIMT treatment [17]. Yet neural changes occurring after bimanual intervention have not been previously described.

The current study sought to characterize brain plasticity following bimanual intervention in children with hemiparesis, via serial behavioral assessment and MR imaging, and to examine the association between brain and behavior changes.

2. Subjects and Methods

The study was approved by the Institutional Review Board and National Research Ethics Committee of the hospital, and fully informed consent was obtained from parents and assent from children.

2.1. Subjects. 12 children with hemiparesis (6 females, mean age 11 ± 3.6 years) were recruited from the Pediatric Neurology Unit at the Tel Aviv Sourasky Medical Center and associated Child Development Centers to a magic-themed HABIT intervention in 2011. The subjects in the current study are part of a larger cohort of CH [6]. Only children with longitudinal imaging were included. Additional inclusion criteria were clinical signs of spastic hemiparesis, attendance in mainstream education, and independence of mobility. Exclusion criteria were any overt seizure activity, initiation of motor therapy or musculoskeletal treatment in the last 6 months, prior surgical intervention, and contraindication to MRI. Level of mobility and functional capacities of the children were confirmed via the Gross Motor Function Classification System (GMFCS) [18] and Manual Ability Classification System (MACS) [19] where higher scores represent greater restrictions to mobility and function. Children were included if GMFCS ≤ II and MACS ≤ III with skills ranging from more mild physical restrictions in mobility and handling of objects to considerable difficulty, thus may use mobility aids and require help to prepare and or modify activities.

2.2. Study Set-Up. Overall 12 children underwent the Magic intervention program.

Eight children participated in the Magic HABIT day camp intervention (60 hours over 2 weeks) and were assessed for hand motor function on 3 occasions: (1) before intervention, (2) immediately after intervention, and (3) 6 weeks following intervention. Six of them underwent 3 MRI scans at the same time points (see Figure 1). The remaining two children (subjects 2 and 8) underwent only two MRI scans (before and immediately following intervention). Four children participated in an outreach home-based Magic HABIT program (a weekly clinical attendance with 2 hours per day of bimanual training monitored by a weekly diary) for 6 weeks (total of 60 hours) and were assessed for hand motor function on 2 occasions (before and after intervention). These children had only two MRI scans (before and immediately following intervention), except for one child (subject 12) who was excluded after the first scan due to substantial head movements and inability to remain still in the scanner. To set a child friendly atmosphere and improve data quality, training in a mock scanner preceded the MRI scans and during the structural series the children watched an animated movie of their choice. In addition, the child's guardian accompanied the child in all study stages including the scans.

2.3. Hand Motor Function Assessment. Children's motor classification included rating according to the MACS and

GMFCS. The MACS classifies ability to handle objects in important daily activities across a five-point scale; children at level I handle most objects easily and at level V they are severely limited in their ability [19]. The GMFCS is a measure of spontaneous functional mobility [18].

Hand motor function was assessed at each of the time points with 2 performance tests, assessed by trained therapists and a self-report questionnaire:

(1) The Assisting Hand Assessment (AHA; version 4.3): a standardized test of spontaneous use and performance of a weaker/affected hand during bimanual interactions in functional/play based tasks with good reliability and validity [20]. Videos were scored by trained therapists blinded to intervention status.

(2) Jebsen-Taylor Test of Hand Function (JTTHF) [21]: a standardized timed test measuring manual dexterity (modified by eliminating the writing task) with reliability and normative data reported for children [22]. A three-minute limit was set for each task with a maximum overall score of 1080 seconds across the 6 tasks.

(3) The Children's Hand Experience Questionnaire (CHEQ): a 29-item questionnaire exploring independent participation and skilled use of an affected/hemiplegic hand in daily bimanual activities and reported competence and worry/confidence [23].

Hand motor improvement was defined as follows.

AHA: Least Detectable Difference (LDD) was defined as $(1.96 * \sqrt{2} * \text{SEM})$ and was equal to 5 points, representing a clinically meaningful difference for an individual using the Rasch weighted log unit scores. Raw scores are transformed into logits via Rasch analysis to account for different degrees of difficulty of the items. Logits are converted to a 0–100 AHA unit scale with higher scores representing better bimanual skills [20].

JTTHF: percentage of change was determined. Calculation of difference beyond 2 standard deviations of the normative data equated to 20%, representing a clinically meaningful difference.

CHEQ: percentages of change relative to baseline in number of activities performed using 2 hands were calculated and change greater than 20% was considered meaningful [6].

2.4. MRI Scanning. Images were acquired on a 3T GE scanner (GE Signa EXCITE, Milwaukee, WI, USA) with training in a mock scanner prior to the first scan.

The MRI protocol included high resolution anatomical 3D fast spoiled gradient echo sequence (FSPGR) (slice thickness/gap = 1/0 mm; field of view (FOV)/matrix: 240 mm/256 × 256; time to repeat (TR)/time to echo (TE) = 8.6/3.3 msec); fMRI performed with T_2^*-weighted gradient echo echo-planar imaging (GE-EPI) sequence (slice thickness/gap = 3.5/0.3 mm; FOV/matrix = 240 mm/128 × 128; TR/TE/flip angle = 2,250/29 msec/79°); DTI acquired along 19 diffusion gradient directions (b = 1000 sec/mm^2) and one with no applied diffusion gradient (slice thickness/gap = 3/0 mm; FOV/matrix = 220 mm/128 × 128; TR/TE = 11,000/91 msec).

2.5. MR Analysis

2.5.1. MRI Motor Paradigm. A block-design fMRI motor task was used in which children clenched and extended all fingers of one hand in synchrony with 2 Hz paced tones. The total task duration was 4 minutes and 48 seconds. There were 18 seconds of silence with one alert beep before the task began to let the children prepare for the motor task, followed by alternations between six epochs of rest, six epochs for right hand, and six epochs for left hand, each lasting 14 seconds. Children were instructed to do their best to move only the affected or less affected hand in isolation. Range of movement was limited to midrange by a soft plastic sponge ball placed in children's palms. Videos were recorded during the fMRI task in order to assess the presence of mirror movements. Mirror movements (MM) were subsequently rated according to the Woods and Teuber scale [24]. On this scale, 0 indicates absence of MM, 1 = barely discernible, 2 = slight but sustained, 3 = strong and sustained, and 4 = movement equal to intended hand.

2.5.2. fMRI Analysis. fMRI analysis was performed with BrainVoyager QX 2 software package (http://www.brainvoyager.com/) and was previously described [9]. Briefly, preprocessing included motion correction (scans with head movement >3 mm were rejected), high-frequency temporal filtering, and removal of low-frequency linear trends. The first six volumes were discarded to allow for stabilization of the signal (to allow for T_2^* equilibration effects). Coregistration was performed between anatomical and functional images. Preprocessed functional images were incorporated into the high resolution anatomical images through trilinear interpolation. The coregistered images were not transformed into a standard space but remained in each subject's native space due to the substantial brain abnormalities in this population. fMRI data sets from the 2 or 3 time points (baseline, after intervention, and follow-up) were coregistered to the 3D FSPGR anatomic sequence of each participant from the baseline scan (T1) to allow comparison between activations at the different time points. Three-dimensional statistical parametric maps were calculated separately for each subject using a general linear model (GLM) in which all stimuli conditions were positive predictors. Two contrasts were studied: contrast 1: affected hand versus baseline and contrast 2: less affected hand versus baseline. We used the false discovery rate (FDR) procedures for the selection of thresholds, and the FDR (q value) chosen in the present study was 0.05.

Two measurements were extracted.

(1) The peak activation in each region of interest (ROI) was detected and a box-shaped volume of 25 voxels was placed around the peak of activation from which beta values were extracted. The beta weights were extracted separately from blocks that included movement of the hand contralateral to the lesion (affected hand) and of the hand ipsilateral to the lesion

(less affected hand). The selected ROI beta weights refer to the fMRI activation level and reflect the level in which each of the predictors explain the signal from the specified region. Therefore, the beta weights characterize the level of task-related activity in each region selected.

(2) We also applied an additional quantitative measure of lateralization index (LI) using the total number of activated voxels for each region of interest. For each ROI, voxels were collected using all conditions and were compared with the baseline condition with a probability value less than 0.05. LI = (contralateral − ipsilateral)/(contralateral + ipsilateral), where contralateral and ipsilateral equal the total number of voxels activated above threshold in areas around the central sulcus contralateral or ipsilateral to the moving hand. This approach yielded LIs for the motor activation around the central sulcus that ranged from +1 for unilateral activation pattern to −1 for ipsilateral activation pattern (atypical), while values close to 0 reflect more bilateral activation patterns.

2.5.3. DTI Analysis. DTI analysis was performed using DTI Studio software (Johns Hopkins University, Baltimore, MD, USA) as previously described [9]. The diffusion tensor was first estimated on a voxel-by-voxel basis and axial diffusivity (Da), radial diffusivity (Dr), mean diffusivity (MD), and fractional anisotropy (FA) maps calculated. The corpus callosum (CC) and corticospinal tract (CST) were reconstructed using streamline fibre tracking with the Fibre Assignment by Continuous Tracking (FACT) algorithm [9]. Fibre tracking was terminated when it reached a pixel with an FA value lower than 0.25 or when the turning angle was >70°. A single ROI was used to extract the CC via a color coded midsagittal FA image [9, 25, 26]. Further segmentation of the CC into genu, midbody, and splenium was performed based on the Witelson parcellation scheme [27]. A multiple ROI approach was used to extract CST tracts, defining fibres that pass from the unilateral pons through the posterior limb of the internal capsule to the motor and premotor cortex [9]. Mean values of Da, Dr, MD, and FA were calculated for each fibre.

Significant change in diffusivity parameters was defined as follows: changes above 5% were considered significant as previous studies reported changes around 5% following learning interventions, with smaller changes likely to reflect natural variation [28, 29].

2.6. Statistical Analysis. Descriptive analysis was performed at a group level to compare the imaging parameters before and after intervention. Improvement on behavioral tests was based on clinical significance as described in Section 2.3 (Hand Motor Function Assessment). For all imaging parameters (beta values, LI, and diffusivity values), mean percent change between pre- and post intervention imaging parameters was computed to assess the change between the different time points and baseline measures. Significant improvement on diffusivity parameters was defined as greater than 5% as described above. Pearson correlations were performed to study the association between the imaging parameters and manual function at the three time points. All statistical analyses were performed using SPSS (Chicago, IL, USA, version 17.0).

3. Results

3.1. Description of Sample. The study group comprised 12 children (6 males) aged 7–16 years (mean 11 ± 3.6 y), of which nine had right hemiparesis and the remainder had left hemiparesis. MACS scores ranged from 1 to 3, while GMFCS scores ranged from 1 to 2. Two children were born preterm and the rest at term. See Table 1 for subject characteristics.

3.2. Behavioral Outcomes. Overall, 11 out of 12 children improved behaviorally after intervention on at least 1 test, and eight children improved on two or more tests (see Table 2). Six out of eight children who were assessed at the third time point maintained improvement at follow-up. It should be noted that the one child who did not improve behaviorally participated in the Magic HABIT camp in the previous year and appeared to experience a ceiling on his hand function progress.

AHA: 7 of 12 children improved significantly (at least 5 points) after intervention. Of these, 3 maintained improvement at follow-up, and 1 further child showed significant improvement only at follow-up.

CHEQ number of independent 2-handed activities: 6 of 12 children improved significantly (at least 20% change) after intervention. Of these, 3 maintained improvement at follow-up, and 1 further child showed significant improvement only at follow-up.

JTTHF reaction time of the affected hand: 8 of 12 children improved significantly after intervention (at least 20% change); 4 children were unable to complete the task within the required time at both time points and thus were scored as not showing improvement. All of the 4 children with the additional assessment at time 3 who improved after intervention maintained the improvement at follow-up.

At the group level, test scores improved after intervention on all tests, with the improvement tapering off at follow-up, apart from the JTTHF where even greater improvement was seen at follow-up (Figures 2(a)–2(c)). Mean increase in AHA scores from pre- to post intervention was 5.13 ± 4.09 points after intervention and 10.68 ± 13.2 points by follow-up, while mean decrease in response time on the JTTHF was 27.48%± 36.44% after intervention and 33.6%± 38.2% from baseline to 6-week follow-up. Mean increase on the CHEQ 2-handed score was 5.58 ± 7.4 points after intervention and 4.57 ± 6.9 points at follow-up.

3.3. Motor fMRI Beta Values across MRI Examinations. Overall, level of activation, as measured by beta values, increased after intervention and continued to increase at follow-up (see Figure 2(d)). Mean change in betas in the affected hemisphere when moving the affected hand (contralateral), from pre- to post intervention was 26.14% increase ($n = 7$) and from preintervention to follow-up was 34.75% increase ($n = 4$).

Table 1: Participant characteristics.

Child	Gender	Age years	Hemiparetic side	GA at birth (weeks)	Birth weight (grams)	Type of injury*	Radiological score	GMFCS	MACS
1	M	7.75	L	29	1298	PVL + focal infarct	6	1	1
2	F	7.9	R	31	2100	Infarct	25	2	2
3	M	13	R	40	2700	Infarct at 2.5 years	28	2	3
4	F	16.25	R	Unknown	Unknown	Vascular infarct	19	1	1
5	F	10.6	R	40	3245	Infarct	7	1	1
6	M	9.5	R	39	3470	Intracranial haemorrhage	18	2	3
7	M	9.75	R	31	2000	IVH	15	2	2
8	M	7.8	R	40	3765	Infarct	13	2	3
Total camp	5/8 males	10.3	7/8 R	35.71	2654		16.38	1.63	2.00
9	F	12.9	L	40	3330	Intracranial haemorrhage from TBI at 3 months	14	2	2
10	F	18.6	R	40	2840	Infarct at 7.5 years	21	2	2
11	M	10.8	R	41	2770	Infarct	10	2	2
12	F	7.5	L	40	—	Intracranial haemorrhage after cardiac surgery at 18 months	16	2	2
Total home intervention	1/4 M	12.45	2/4 R	40.25	2980		15.25	2	2

GA: gestational age; PVL: periventricular leukomalacia; TBI: traumatic brain injury; IVH: intraventricular hemorrhage; MACS: Manual Ability Classification System; GMFCS: Gross Motor Function Classification System. Radiological score was calculated according to Shiran et al. [30]. *Injury occurred pre- or perinatally unless specified otherwise.

Table 2: Individual behavioral scores for all participants before and after treatment and at six-week follow-up.

Type	Case	AHA1	AHA2	AHA3	CHEQ1	CHEQ2	CHEQ3	JTTHF1	JTTHF2	JTTHF3
		Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up
Camp	1	77	80	82≈	25	27	28	152.3	38.2*	29≈
	2	42	41	—	6	17*	—	1080	1080	—
	3	27	32*	37≈	5	15*	15≈	1080	1080	1080
	4	63	72*	63	26	26	22	462.5	48.6*	50≈
	5	71	76*	76≈	20	23	25≈	68.6	52.8*	43≈
	6	46	47	46	17	15	12	1080	1080	1080
	7	55	63*	58	0	18*	12≈	612.5	365*	442.5≈
	8	32	43*	38≈	0	18*	12≈	1080	1080	1080
		Pre	Post		Pre	Post		Pre	Post	
Home	9	52	58*		4	6*		476.1	241.8*	
	10	45	54*		16	13		578.5	355.6*	
	11	59	63		8	17*		258	85.8*	
	12	57	60		24	23		923.9	612.1*	

*Significant change between assessments at pre- and post intervention.
≈Significant change between preintervention and follow-up assessments.
AHA = Assisting Hand Assessment, CHEQ = Children's Hand Experience Questionnaire, and JTTHF = Jebsen-Taylor Test of Hand Function.

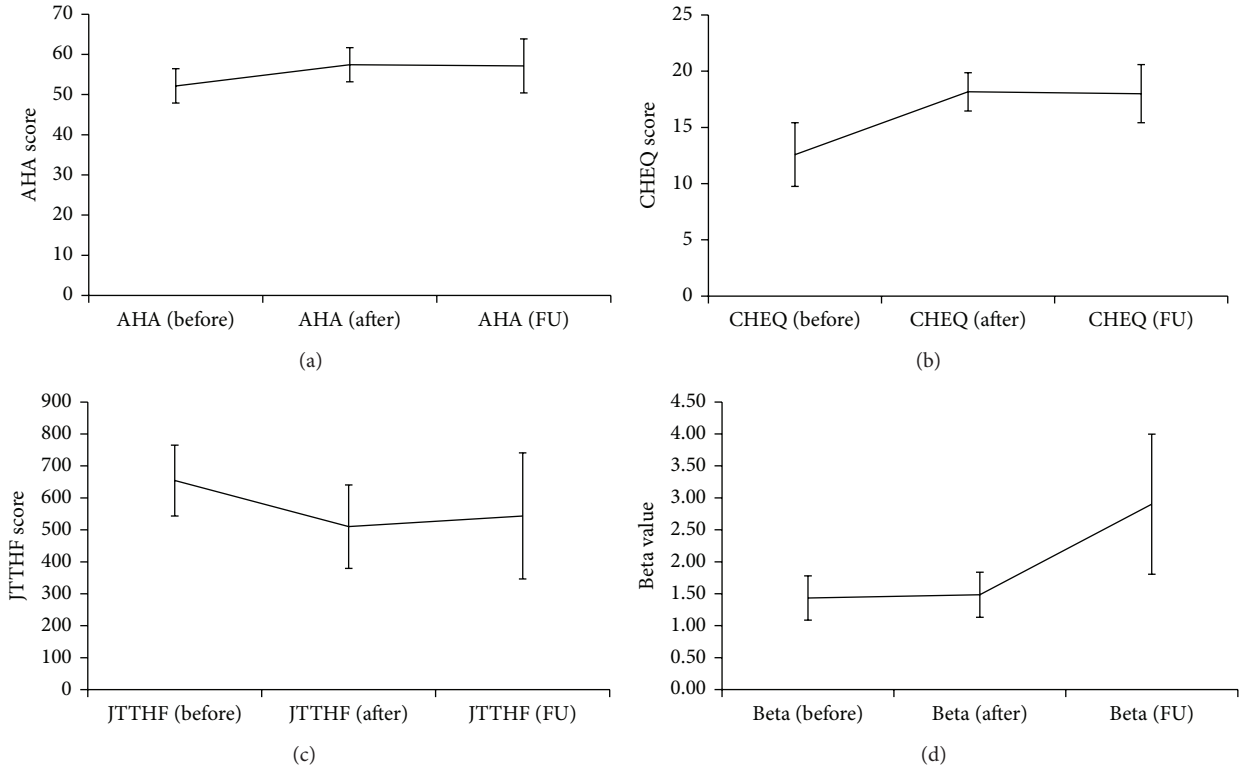

Figure 2: Group means and standard error for (a) AHA, (b) CHEQ number of 2-handed activities, (c) JTTHF of the affected hand, and (d) beta values in affected hemisphere when moving the affected hand. Manual function: before and after, $n = 12$; follow-up, $n = 8$. Betas: before and after, $n = 7$, follow-up, $n = 4$.

In the less affected hemisphere, there was little change in level of activation (ipsilateral to movement of the affected hand) with mean change from pre- to post intervention of −2.4% ($n = 4$). One child (subject 11) was rated as having significant mirror movements (score of 3 on Woods and Teuber scale) which might affect these values. Yet, when excluding this child from the analysis, similar effects were detected with increase of 24% mean percent change from pre- to post intervention (compared to 26.14%) in the affected hemisphere when moving the affected hand and +10% change in the less affected hemisphere when moving the affected hand (compared to −2.4%). There was similarly little change in activation in the less affected hemisphere when moving the less affected hand (contralateral) of −1.5% ($n = 8$) after intervention and 7.6% ($n = 5$) change by follow-up. Only one child had pre- and post intervention activation in the ipsilateral hemisphere when moving the less affected hand.

The most severe case of hemiplegia (case 6), as represented by MACS 3, was the one child who did not respond behaviorally to the intervention. Despite the absence of functional improvement, increases in brain activity as measured by beta levels during hand movement were seen.

3.4. Motor fMRI: LI across MRI Examinations. Motor related activation was seen in the sensorimotor areas around the central sulcus and in the supplementary motor areas (SMA). In general, a shift to a unilateral activation after intervention was detected in some children when moving the affected hand. LI when moving the less affected hand remained stable in all children except one (subject 7), who moved to a more unilateral pattern. Of the 11 children who improved behaviorally, 4 showed improvements in LI on movement of the affected hand, shifting towards a unilateral pattern, or maintained an originally unilateral pattern of activation (subjects 1, 2, 4, and 5) (see Table 3). These improvements were maintained at follow-up (except for 1 child who had no follow-up scan). One child showed improved LI only at follow-up (subject 6). For three children, the LI could not be calculated due to poor data quality of the fMRI scan (subjects 3, 8, and 12). Three children improved behaviorally yet did not have increased LI after intervention, with some demonstrating a pattern of ipsilateral activation (subjects 7, 10, and 11). Figure 3 shows a graphic presentation of activation in three children, before and after intervention. Subjects 4 and 5 show a change towards unilateral activation after intervention, while only a slight change was evident in subject 6.

3.5. Correlations between LI When Moving the Affected Hand with Manual Function at the 3 Time Points. To test the hypothesis that the higher the LI (the more typical/unilateral the pattern of activation), the better the manual function, a correlation analysis between LI and performance was conducted at the 3 time points. Overall, the analysis showed that higher LI values after intervention correlated with better manual skills. A borderline significant correlation ($r = 0.62$,

Table 3: Lateralization index for all participants across examinations.

Case	Affected LI			Less affected LI			Behavioral improvement
	Before	After	FU	Before	After	FU	
1	1	1	1	1	1	1	UM + BM
2	0.4	0.74	—	1	1	—	BM only
3	—	—	—	—	—	—	BM only
4	−0.02	1	1	1	1	1	UM + BM
5	0.8	1	0.91	1	1	1	UM + BM
6	0.055	−0.37	1	0.97	0.97	0.94	None
7	−0.83	−1	−0.16	0.4	1	1	UM + BM
8	—	—	—	—	—	—	BM only
9	−1	—	—	1	—	—	UM + BM
10	1	−0.27	—	1	1	—	UM + BM
11	0.28	−1	—	0.46	0.41	—	UM + BM
12	—	—	—	—	—	—	UM only

LI: lateralization index; FU: follow-up (6 weeks after intervention); UM: unimanual; BM: bimanual.

$p = 0.056$) was detected between LI before intervention and preintervention performance on the CHEQ and at post intervention this correlation was significant ($r = 0.686$, $p = 0.041$) (see Figure 4). Similar relationships were not evident between LI and AHA or JTTHF at pre- and immediately post intervention. At follow-up, there was very little variance in LI (4/5 children had values at or close to 1) hampering correlation analysis. Therefore, we examined the correlation between LI after intervention and manual function at follow-up, which enabled us to assess correlation after intervention, either immediately after or at follow-up. In this analysis, strong correlations were detected with all behavioural measures at follow-up: AHA ($r = 0.820$, $p = 0.046$), CHEQ ($r = 0.941$, $p = 0.005$), and JTHHF ($r = -0.814$, $p = 0.049$) (see Figure 4).

3.6. DTI Changes in the CC and CST across MRI Examinations. At the group level, no significant changes were detected for the MD and FA values before and after intervention in the CC and in the affected and less affected CST. All changes were under 5% (natural variation). However, on an individual level, several children showed post intervention changes in DTI values associated with improved WM integrity in the CC, affected, and less affected CST greater than those to be expected with natural variation (see Tables 4 and 5). Seven children showed improved diffusivity values in the CC after intervention. One child showed improvements in all segments of the CC. In the CST, 3 children showed improved diffusivity values on the affected side after intervention and 5 children on the less affected side.

3.7. Correlations between WM Integrity at the CC and CST with Manual Function at the 3 Time Points. Overall, both before and after intervention, increased WM integrity was related to better hand function. Before intervention, significant correlations were detected between higher WM integrity in the genu and midbody of the CC and better baseline manual function. Lower MD in the genu and midbody was related to higher AHA scores ($r = -0.58$, $p = 0.05$; $r = -0.75$, $p = 0.008$; resp.). Lower MD and higher FA in the genu, midbody and splenium were related to better performance on the JTTHF (genu: FA $r = -0.618$, $p = 0.032$, midbody: MD $r = 0.668$, $p = 0.025$, FA $r = -0.675$, $p = 0.023$; splenium: MD $r = 0.578$, $p = 0.049$; resp.). Higher WM integrity in both the affected and less affected CST was correlated with better unimanual function (JTTHF and FA in the less affected CST: $r = -0.615$, $p = 0.033$; JTTHF and affected CST: FA $r = -0.667$, $p = 0.035$; MD $r = 0.664$, $p = 0.036$).

After intervention, higher WM integrity in the midbody of the CC (reflected by low MD and high FA) was associated with better bimanual function (AHA: MD: $r = -0.815$, $p = 0.004$; FA: $r = 0.670$, $p = 0.034$) and with better unimanual function (JTTHF: MD: $r = 0.772$, $p = 0.009$; FA: $r = -0.687$, $p = 0.028$). No significant correlations were detected between WM integrity and the CHEQ. At follow-up, FA in the genu of the CC was significantly correlated with AHA (FA: $r = 0.85$, $p = 0.03$) and both FA and MD with CHEQ 2 hands (FA: $r = 0.90$, $p = 0.037$; MD: $r = -0.93$, $p = 0.007$). MD and FA in the midbody of the CC were also correlated with AHA (MD: $r = -0.97$, $p = 0.006$; FA: $r = 0.95$, $p = 0.012$) and JTTJF (MD: $r = 0.93$, $p = 0.021$; FA: $r = -0.88$, $p = 0.048$). No significant correlations were detected between WM integrity of the CST and manual function at either post intervention or follow-up.

4. Discussion

This study shows the first evidence of brain plasticity in CH following bimanual intervention. Children underwent serial MRI scans including fMRI and DTI and behavioral assessments. Results from this study show changes in levels of activation, in pattern of lateralization, and in WM integrity following intervention. In addition, such changes were correlated with behavioral assessment at all three time points shedding light on possible pathways to explain how behavioral improvement following bimanual intervention is manifested in the brain. Nevertheless, it is important to note that these changes were not detected in all CH regardless of the behavioral gains they showed.

A main finding of this study is a shift towards a more unilateral activation pattern after intervention, reflected by higher LI values. At the group level, abnormal pattern of brain activation was detected at baseline reflected by bilateral activation. In typically developing subjects, motor activation is primarily unilateral, being limited to the hemisphere contralateral to the hand in movement [9]. Following intervention, increased level of activation in the affected hemisphere was detected (manifested by increased beta values) in parallel with the shift in lateralization. We interpreted these findings as indicating neuroplasticity towards a more typical brain activation pattern. These results are in line with a previous study that reported change in LI towards unilateral pattern after CIMT treatment in a small sample ($n = 4$) [17]. A recent systematic review also described several types of brain changes following therapy such as an increase in M1

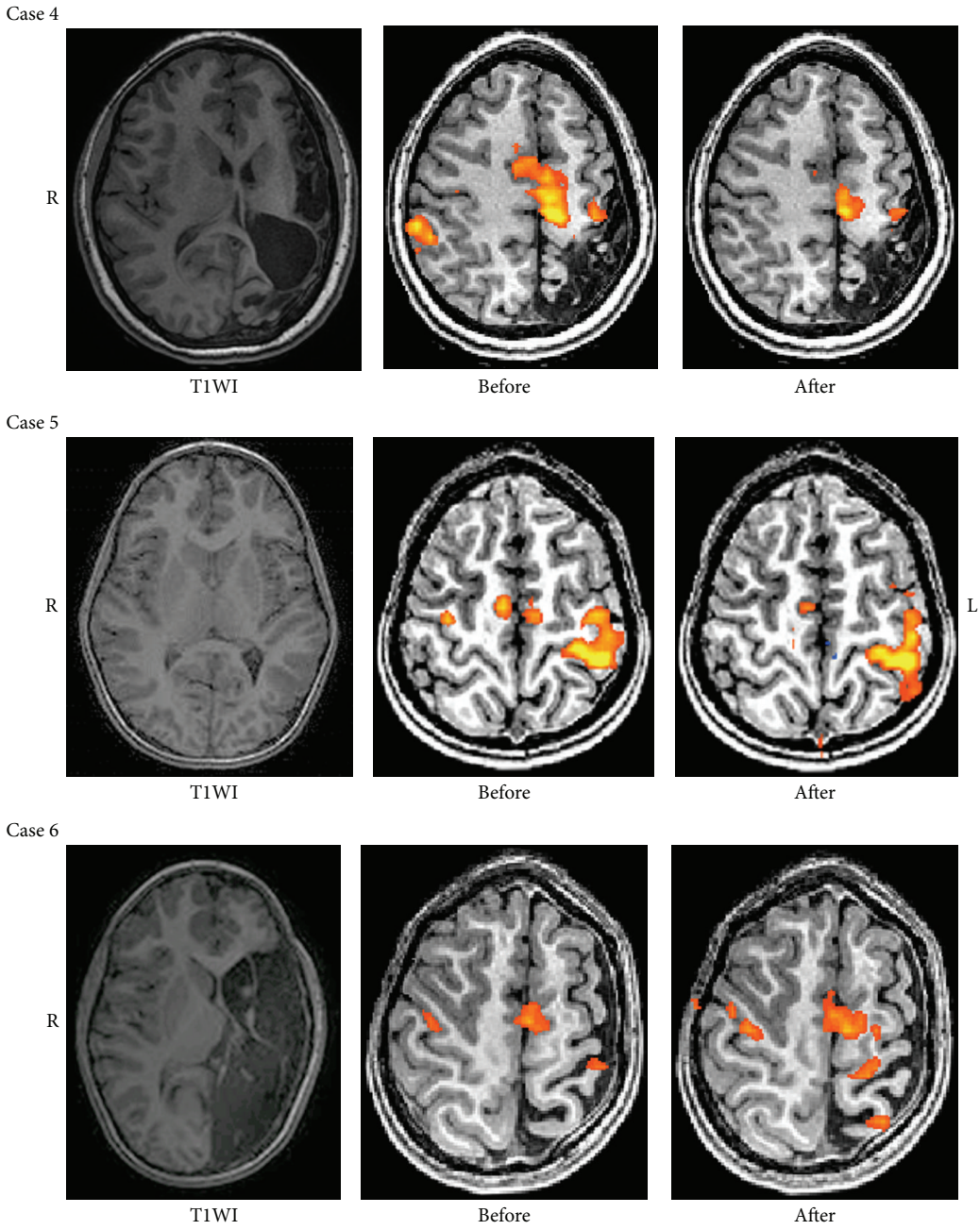

Figure 3: Examples of fMRI motor activation in areas around the central sulcus and supplementary motor area (SMA) for the condition of moving the affected hand. T1WI = T1 weighted imaging. In cases 4 and 5, a more unilateral pattern of activation is seen after intervention. In case 6, there is more activation in the affected hemisphere after intervention.

excitability in subjects with ipsilesional reorganization and a decrease in M1 excitability in subjects with contralesional reorganization [10], indicating treatment-related plasticity. However, brain plasticity is a complex process and changes and varying etiologies, brain injury subtypes, or developmental experiences may have differential effects on neuroplastic changes following intervention. Further studies are needed to address these issues.

The association between LI and manual function became stronger after the intervention with additional associations (with both unimanual and bimanual functions) emerging 6 weeks following intervention. Yet, the improvement in LI at follow-up was not matched by further behavioral improvements at this time. This may reflect the consolidation time of the newly learned skills (plasticity processes) enabling detectable expressions of relations between newly strengthened brain networks and more effective manual function by the end of treatment, and even more so at follow-up. Indeed, children were encouraged to keep practicing their newly learned manual skills; therefore, it may be that the plasticity

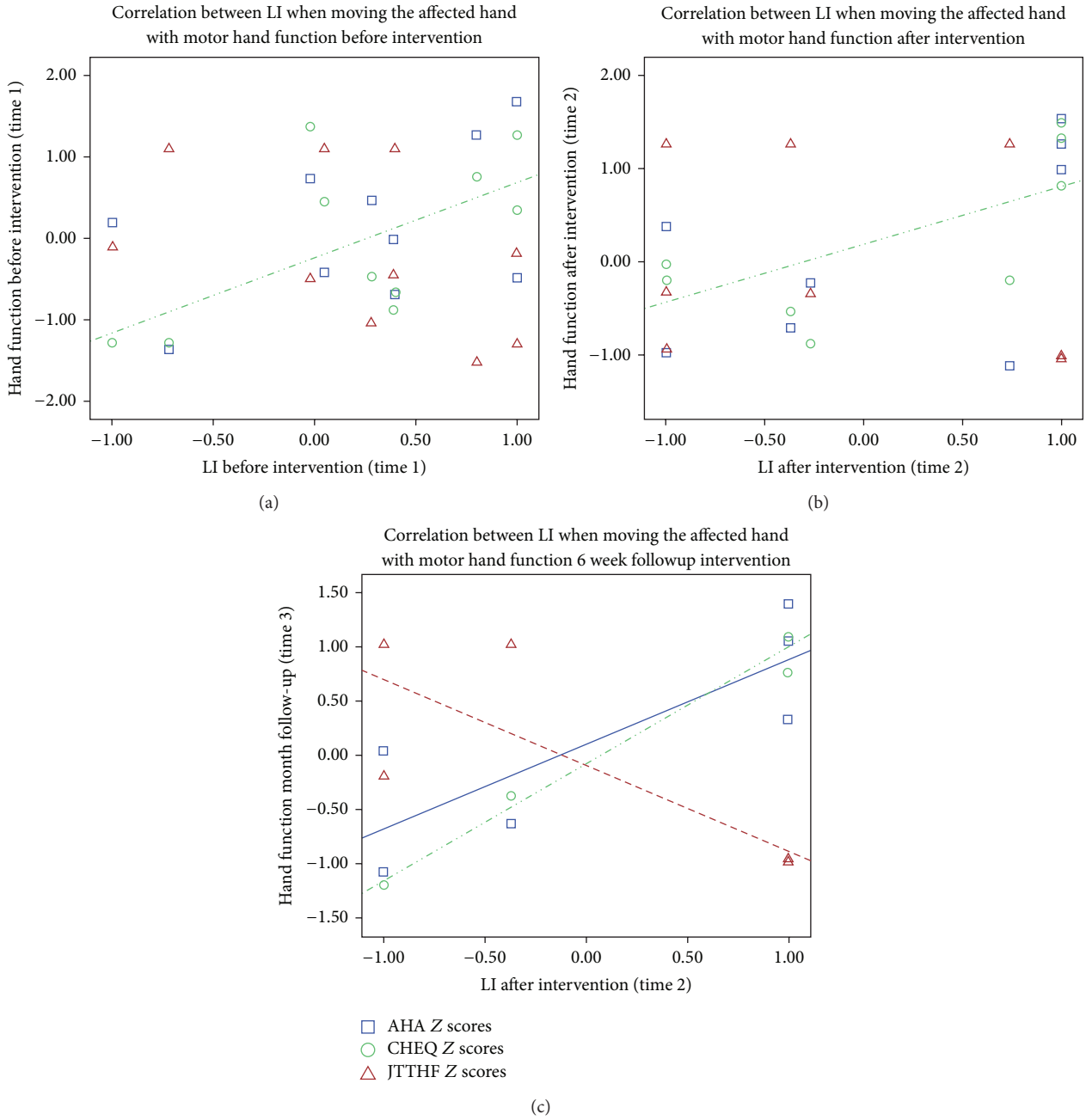

Figure 4: (a) Correlations between LI and manual function at (a) time 1: before intervention, (b) time 2: immediately after intervention, and (c) LI at time 2 and hand function at follow-up. Y-axis presents AHA, CHEQ, and JTTHF Z scores.

processes continued during the six weeks enabling greater efficacy in more varied contexts.

The finding that the associations between brain function and structure with behavior were more evident at follow-up may support the dynamical systems theory [31] which postulates that there is a period of instability evident in changing neural networks. From the motor perspective, there are different timescales in the characterization of changing behavior which is reflected in motor learning and development, demonstrated by different learning curves [32]. Nikolai Bernstein's theory of the "degrees of freedom" also relates to a period of enhanced variability in motor learning before the emergence of smooth dynamic motor control [33, 34].

At the group level, no changes in WM integrity of large fibre tracts were seen following intervention, although more than half of the children showed significant change in at least one WM fibre tract in at least one diffusivity parameter. A threshold of 5% was chosen as a significant change based on several studies [28, 29]. Scholz et al. [29] reported on a 6-week juggling intervention in young adults and reported mean increases in FA after training in the order of 5% compared to baseline, with controls showing no significant change.

Table 4: Diffusivity parameters in the corpus callosum: before and after intervention and at six weeks following bimanual intervention.

Case	Genu						Midbody						Splenium					
	MD ($\times 10^{-3}$ mm^2/s)			FA (a.u.)			MD ($\times 10^{-3}$ mm^2/s)			FA (a.u.)			MD ($\times 10^{-3}$ mm^2/s)			FA (a.u.)		
Camp intervention	Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up
1	0.85	0.86	0.85	0.63	0.61	0.62	0.85	0.82	0.83	0.59	0.61	0.59	0.8	0.79	0.82	0.67	0.68	0.66
2	1.02	1.05	—	0.56	0.57	—	1.07	1.06	—	0.48	0.48	—	1.12	1.16	—	**0.49**	**0.56***	—
3	**1.01**	**1.02**	**0.95***	0.57	0.57	0.57	**1.24**	**1.00***	**1.06***	**0.48**	**0.52***	**0.44**	**0.96**	**0.90***	0.99	0.63	0.63	0.59
4	0.89	0.88	0.89	0.6	0.6	0.59	**0.93**	**0.87***	**0.88***	0.58	0.58	0.56	1.04	1.02	1.19	**0.54**	**0.58***	**0.57***
5	0.84	0.86	0.86	0.61	0.61	0.6	0.78	0.79	0.82	0.62	0.61	0.59	0.8	0.8	0.83	0.66	0.65	0.64
6	**0.92**	**0.85***	0.9	**0.54**	**0.59***	**0.58***	0.94	0.93	0.98	0.49	0.5	0.52	**1.11**	**1.06***	**1.01***	0.47	0.43	0.45
7	0.97	1.01	1	0.6	0.63	0.57	**	**	**	**	**	**	**0.95**	**0.86***	**0.87***	0.67	0.69	0.68
8	0.91	0.92	—	0.59	0.6	—	0.93	0.92	—	0.6	0.6	—	0.84	0.91	—	0.7	0.69	—
Home intervention	Before	After		Before	After		Before	After		Before	After		Before	After		Before	After	
9	0.92	0.99		**0.61**	**0.56***		0.89	0.85		0.57	0.58		**1.04**	**0.95***		**0.56**	**0.60***	
10	0.95	0.92		0.59	0.6		0.84	0.86		0.62	0.59		0.75	0.75		0.7	0.7	
11	0.9	0.96		0.59	0.59		**1.1**	**0.93***		0.55	0.57		0.82	0.81		0.67	0.69	
12	—	—		—	—													

MD = mean diffusivity ($\times 10^{-3}$ mm^2/s); FA = fractional diffusivity (arbitrary units).

*Significant improvement >5% from baseline.

**Due to large lesion size, we were unable to reconstruct this segment.

Table 5: Diffusivity parameters in the affected corticospinal tract at 3 time points.

Case	Affected CST						Less affected CST						Behavior improv.
	MD ($\times 10^{-3}$ mm^2/s)			FA (a.u.)			MD ($\times 10^{-3}$ mm^2/s)			FA (a.u.)			
	T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3	
Camp intervention	Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up	Before	After	Follow-up	
1	**0.79**	**0.75***	0.76	0.59	0.6	0.59	0.77	0.74	0.76	0.61	0.62	0.61	Yes
2	**	**	**	**	**	**	**0.8**	**0.75***	—	**0.56**	**0.62***	—	Yes
3	0.8	0.84	0.81	0.55	0.55	0.55	**0.8**	**0.75***	0.8	**0.54**	**0.58***	0.56	Yes
4	0.79	0.85	0.84	0.58	0.6	**0.62***	**0.88**	**0.75***	**0.77***	**0.62**	**0.58**	**0.57***	Yes
5	0.73	0.74	0.78	0.61	0.58	0.6	0.72	0.71	0.75	0.6	0.6	0.6	Yes
6	**	**	**	**	**	**	0.76	0.74	0.74	0.59	0.58	0.6	No
7	**0.84**	**0.76***	0.82	**0.6**	**0.66***	0.62	0.74	0.73	0.75	0.62	0.6	0.65	Yes
8	0.89	0.87		0.56	0.56		0.73	0.76		0.62	0.62		Yes
Home intervention	Before	After		Before	After		Before	After		Before	After		
9	**0.83**	**0.77***		**0.6**	**0.65***		**0.76**	**0.71***		**0.61**	**0.65***		Yes
10	0.79	0.82		0.62	0.58		**0.79**	**0.71***		0.65	0.61		Yes
11	0.81	0.81		0.62	0.6		0.75	0.76		0.63	0.6		Yes
12	—	—		—	—		—	—		—	—		Yes

MD = mean diffusivity ($\times 10^{-3}$ mm^2/s); FA = fractional diffusivity (arbitrary units). *Significant improvement = >5% improvement. **Due to large lesion size, we were unable to reconstruct this tract.

Similarly, a study of young adults learning a new language [28] reported up to 5% change in FA in learners versus controls over a 9-month learning period. Changes in FA in language areas were 1-2% after 1 month, rising to 5% after 9 months; however, controls also showed up to 3-4% change in FA, therefore limiting the reliability of the detected changes. It may be that DTI, despite its sensitivity and important value in learning about WM integrity, is still too crude to measure subtle microstructural changes that may take place following a 2-week intervention. Other diffusion based methods with higher spatial resolution and higher b value may be more sensitive to detect such changes [35].

Importantly relationships between WM integrity and manual functions emerged as a function of the intervention, and these relations remained significant and became stronger throughout the study period. Mainly mean diffusivity in the midbody of the CC was associated with better bimanual (AHA) and unimanual (JTTHF) skills. MD values in the midbody of the CC were correlated with the CHEQ at follow-up, and this association was not detected before intervention and was not yet evident directly after intervention. Indeed the CHEQ, which reflects changes in bimanual function in daily activities, was previously reported to significantly improve after intervention [6].

There are several methodological challenges when conducting imaging research in pediatric populations with some challenges specific to CH. There are often problems with data quality caused by excess movements, since children often find it difficult to remain still in the magnet. In addition, many children with CH exhibit additional comorbidities such as attention deficit hyperactivity disorder [36, 37]. Thus, scanning children, especially CH, requires a special set-up. In this study, we used a special set-up which included a practice session in a mock scanner; the presence of the child's guardian during all study stages including the MRI scan; and watching an animation movie of the child's choice during the structural series of the scan. In the current study, we had to exclude only a few data sets due to motion artefacts, but in general the special set-up improved the children's cooperation and data quality.

An additional challenge is that children with movement difficulties frequently show associated head movements with the effort of moving their hands resulting in further motion artefact. Furthermore, the phenomenon of mirror movements which are frequently observed in CH may influence fMRI motor activation measurements. In our study, we recorded videos of the children when performing the motor task and retrospectively could identify mirror movements and take them into account during analysis. Only one child demonstrated significant mirror movements and excluding him did not have a major influence.

Finally, there are methodological challenges in conducting longitudinal MRI studies that quantitatively compare scans acquired at different times. There are many parameters that may affect the signal such as different level of head movement and different level of cooperation and grip force and parameters relating to the magnet. We tried to overcome these problems by using FDR for statistical analysis and by using the laterality index which provides a type of normalization of the fMRI data.

There are several limitations in this study, with some that are inherent to studies of CH. In this study, we had a relatively small sample size since it is difficult to recruit this population and since all children were enrolled to

an intervention program which required attendance to a 2-week camp or adherence to a home-based programme. The heterogeneity of the sample due to the varied etiologies underlying CH may affect both the clinical motor features of the children and the type of brain pathology, making it difficult to find a general pattern of plasticity following intervention in this population. Another limitation lies in the hand function assessments that may have impeded our ability to detect change. In particular, we noted a ceiling effect on the JTTHF in which some capacity may have been demonstrated, but unless all items within each task were completed successfully (e.g., all 5 cards turned), a maximum time score of 1080 seconds was awarded and therefore not reflective of more discrete changes.

In conclusion, changes in DTI and fMRI parameters were seen when comparing pre- and post intervention scans in CH following HABIT. Brain plasticity varied in the study group with children showing different patterns of change after intervention. However, change towards a more unilateral brain activation pattern was consistently associated with motor improvements, thereby adding evidence of measurable neuroplasticity changes following bimanual intervention in children with hemiparesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Maya Weinstein and Vicki Myers are equal contributors.

Acknowledgments

The authors are grateful to the children and their families who gave so much of their time to participate in this study. They would also like to thank physiotherapy and occupational therapy students from Tel Aviv University, Becca Krom, Ron Schertzi, and the Israeli Society of Magicians for their support. This project was funded by grants from Guy's and St Thomas' Charity, Marnie Kimelman Trust and ILAN, the Israeli Association for Disabled children. Beit Issie Shapiro funded and provided the camp venue. D. Green was supported by a grant from the Department of Immigration and Absorption during 2010-2011.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 389520, 12 pages
http://dx.doi.org/10.1155/2015/389520

Research Article

The Morphofunctional Effect of the Transplantation of Bone Marrow Stromal Cells and Predegenerated Peripheral Nerve in Chronic Paraplegic Rat Model via Spinal Cord Transection

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Received 30 January 2015; Revised 27 May 2015; Accepted 7 June 2015

Academic Editor: John Gensel

Functional recovery following spinal cord injury (SCI) is limited by poor axonal and cellular regeneration as well as the failure to replace damaged myelin. Employed separately, both the transplantation of the predegenerated peripheral nerve (PPN) and the transplantation of bone marrow stromal cells (BMSCs) have been shown to promote the regrowth and remyelination of the damaged central axons in SCI models of hemisection, transection, and contusion injury. With the aim to test the effects of the combined transplantation of PPN and BMSC on regrowth, remyelination, and locomotor function in an adult rat model of spinal cord (SC) transection, 39 Fischer 344 rats underwent SC transection at T9 level. Four weeks later they were randomly assigned to traumatic spinal cord injury (TSCI) without treatment, TSCI + Fibrin Glue (FG), TSCI + FG + PPN, and TSCI + FG + PPN + BMSCs. Eight weeks after, transplantation was carried out on immunofluorescence and electron microscope studies. The results showed greater axonal regrowth and remyelination in experimental groups TSCI + FG + PPN and TSCI + FG + PPN + BMSCs analyzed with GAP-43, neuritin, and myelin basic protein. It is concluded that the combined treatment of PPN and BMSCs is a favorable strategy for axonal regrowth and remyelination in a chronic SC transection model.

1. Introduction

Traumatic spinal cord injury (TSCI) causes permanent disability characterized by paralysis and loss of sensitivity as well as multiple metabolic and systemic alterations associated with the dysfunction of the autonomic nervous system [1].

Until now, there is no effective treatment for both acute and chronic SCI, despite several strategies that have been carried out to promote regeneration and improve function. Within these strategies, the use of tissue transplantation has been proposed. Due to its organized structure, the use of peripheral nerve acts as a physical guide via which axons

are encouraged to grow [2]. They are also distally connected and act as a neuroprotector for the preserved spinal cord [2–6]. Furthermore, due to the action of the Schwann cells and macrophages stemming from the PPN, the regeneration and axonal remyelination are supported, since they are capable of secreting growth factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), and granulocyte-macrophage colony-stimulating factor (GM-CSF) which promote neuronal survival [7–13].

Another strategy employed is the use of adult bone marrow stromal cells (BMSCs) since they have the capacity for self-renewal and differentiation. It has been demonstrated that the use of BMSCs in TSCI assists in modulating the central nervous system (CNS) environment to promote its repair and secreting anti-inflammatory and antiapoptotic molecules and growth factors, which promote axonal growth, immunomodulation, angiogenesis, remyelination, and protection from cell death caused by apoptosis [14]. They have been shown to have the capacity to differentiate into different neural linages both in vitro and in vivo, including neurons, astrocytes, oligodendrocytes, Schwann cells, and microglia [15, 16]. Furthermore, they promote axonal regrowth, remyelination, and the improvement of locomotor function, since they are capable of secreting growth factors such as BDNF, NT-3, VEGF, and bFGF [17–22].

As well as those previously mentioned, there are multiple strategies that have been observed to promote axonal regrowth and the reworking of the central nervous system (CNS) in mammals that have suffered a TSCI. However, none of these alone has been able to reestablish total functionality of the injured spinal cord (SC). As such, it is feasible to believe that the use of two of these in conjunction would produce greater functionality.

The objective of this study was to evaluate the morphological and functional effect of transplanting BMSCs and PPN into chronic paraplegic rat model that has undergone complete spinal cord transection. Our hypothesis was that the combination of PPN and BMSCs would give better results compared to those obtained from untreated rats or rats treated with individual transplants.

2. Materials and Methods

2.1. Experimental Design. This study was authorized by the Research and Ethics Committee of the Hospital de Especialidades, Centro Medico Nacional Siglo XXI, Instituto Mexicano del Seguro Social (IMSS); use, handling, and care of animals were carried out following the Official Mexican Standard (Norma Oficial Mexicana) NOM-062-ZOO-1999, which is supported on international standards. A total of 84 Fischer 344 rats were used, aged between 8–10 weeks and weighing between 200 and 220 g. For the TSCI and transplant procedures, 39 females were used having been divided into four random groups (immunofluorescence and histology: Control group: 7 animals; fibrin glue group: 8 animals; PPN group: 12 animals; PPN + BMSCs: 12 animals; electron microscopy: 3 animals per group), 25 males were used as sciatic nerve donors, and 20 males were used to obtain BMSCs.

2.2. Surgical Procedures and Transplant Preparation

2.2.1. Spinal Cord Injury. In order to produce the spinal injury, the rats were anaesthetized by the intraperitoneal injection of a mixture of ketamine (70 mg/kg) and Xylazine (10 mg/kg). A laminectomy was carried out at T9 before a sagittal section was made to the dural sac on the dorsal side. A complete transection of the exposed spinal cord was carried out immediately using microsurgery scissors; finally, the surgical wound was stitched using layered closure.

2.2.2. Preparing the Peripheral Nerve for Transplantation. Twenty-one days before the transplant, the peripheral nerve donor rats were anaesthetized before undergoing a complete transverse section of the sciatic nerve in the upper part of the thigh; the caudal stump of the sectioned nerve was fixed to the surrounding muscle with a 5-0 nylon suture. On the day of the transplant, the rat was anaesthetized to extract a segment of sciatic nerve distal to the cut of approximately 2 cm in length. The nerve fragment was placed in chilled isotonic saline solution until the time of transplantation.

2.2.3. Preparing the Cells for Transplantation. The BMSCs were obtained from rats euthanized with an overdose of sodium pentobarbital. The bone marrow was obtained from both femurs and tibias using a 200 μL micropipette and deposited in a 15 mL conical tube with culture medium (Dulbecco's Modified Eagle (DMEM) GIBCO). Following this, the sample was centrifuged at 1500 rpm for 7 minutes. The cells were then separated using a Ficoll (SIGMA) (3 mL) gradient centrifuged at 2000 rpm at 24°C for 30 minutes. The total number of nucleated cells obtained was quantified and 9×10^6 cells were seeded into a 75 cm^2 culture flask (in 5 mL of DMEM with 20% fetal bovine serum (FBS), from GIBCO), 1 mL of L-Glutamine (GIBCO), 5 mL of HEPES (SIGMA), and 1 mL of Penicillin-Streptomycin (GIBCO); they were then placed in a water-jacketed incubator at 37°C with 5% CO_2 until the cells formed a fibroblast monolayer. Finally, the BMSCs were reseeded onto the fibroblast layer and maintained for two weeks until transplantation time.

2.2.4. Phenotypic Classification of BMSCs. Flow cytometry was used to classify the mature BMSCs. The cells were centrifuged at 1500 rpm for five minutes and they were incubated with primary antibodies (Cd117 Millipore; Cd13 Santa Cruz Biotechnology Inc; Cd34 Santa Cruz Biotechnology Inc, all at a dilution of 1 : 100) in darkness for 20 minutes at 4°C. They were washed twice with FACS Buffer before being centrifuged again at 1500 rpm for five minutes. The cells marked with Cd117 were incubated in darkness for two hours with the secondary antibody (Alexa 488 or 586, Molecular Probes Invitrogen 1 : 200); they were then fixed in 4% paraformaldehyde for 1 hour before finally being quantified and analyzed with flow cytometry using the CellQuest Pro (BD Biosciences) program. Cell phenotype proportion is shown in Figure 1. 80.45% of the cells were positive for Cd13 (marker for subpopulation of mesenchymal stem cells); 11.49% were positive for Cd117 (marker for subpopulation of mesenchymal stem cells); and 10.69% of the

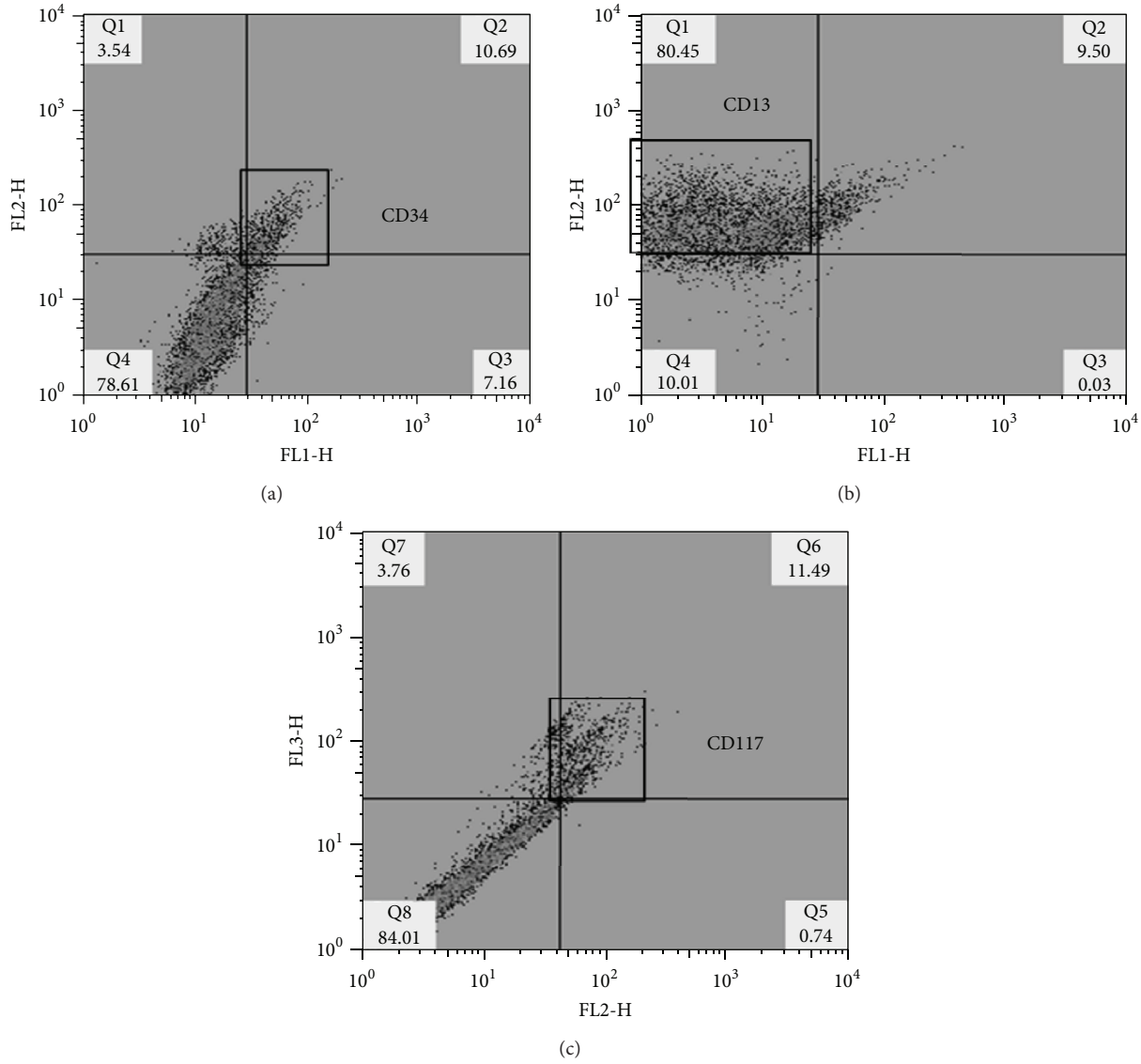


Figure 1: Bone marrow stromal cells (BMSCs) flow cytometry with Cd13 and Cd117. (a) Population of Cd34-positive cells (10.69%). (b) Population of Cd13-positive BMSCs (80.45%). (c) Population of Cd117-positive BMSCs (11.49%).

cellular population was positive for Cd34 (specific control marker for hematopoietic stem cells). As such, the majority of cells transplanted were adult mesenchymal stem cells.

2.2.5. Transplant. Four weeks after the spinal cord transection, the rats were assigned to one of four experimental groups. In Group 1 (Control, $n = 7$), the surgical wound was reopened enough to expose the dural sac. In Group 2 (positive Control $n = 8$), the dural sac was reopened and the scar was carefully removed from the spinal cord and the end of the medullary stumps, leaving a cavity of approximately 6 mm of amputated length; the cavity was filled with fibrin glue (BAXTER). In Group 3 ($n = 12$), the same procedure was carried out as described for group 2, except, in this case, 3 or 4 segments of the sciatic peripheral nerve, each of approximately 6 mm in length, were transplanted lengthways into the medullary cavity; the implants were fixed with fibrin glue (BAXTER). In Group 4 ($n = 12$), in addition to the procedures detailed for group 3, BMSCs were transplanted via four injections, two in the proximal medullary stump and two in the distal medullary stump, in the center of each hemicord; each injection contained 3×10^4 cells in 5 μL of Hank's solution (SIGMA).

2.3. Evaluations

2.3.1. Motor Function Evaluation. Hindlimb locomotion was evaluated using the Basso, Beattie, Bresnahan (BBB) Locomotor Rating Scale [23]. The scale of the BBB evaluates the movements of the hindlimb of the animals; the scale oscillates between 0–21 points, where 0 is no movement and 21 is a normal movement of the hindlimbs. The evaluator

was unaware of the treatment assigned to each animal. The animals were evaluated 24 hours after the transplant and every two weeks during the following 8 weeks.

2.3.2. Processing of Samples for Immunofluorescence. Eight weeks after transplantation, the animals were anaesthetized with sodium pentobarbital (40 mg/kg i.p) before being perfused by intracardiac injection with 4% paraformaldehyde. A 2 cm long segment was obtained from the spinal cord with the injury zone in the center. The samples were placed in a 30% sucrose solution in PBS for 24 hours; 12 μm thick sagittal frozen serial sections were then cut on a LEICA CM1510S cryostat. Following this, the sections were incubated for 48 hours at 4°C with the primary antibodies (Protein basic myelin (PBM) D18; Neuritin FL-142, and Gap-43 H-100, Santa Cruz Biotechnology Inc.). The sections were later washed with PBS and incubated for 2 hours with the secondary antibody (Alexa 488 Anti-Rabbit or Anti-Mouse, Molecular Probes Invitrogen); they were washed with PBS and stained using propidium iodide (red nuclei) for 1 minute. Finally, they were covered with Vectashield (Vector Labs) in order to be analyzed with a fluorescence microscope (Carl Zeiss). For each specimen, 6 photos were taken from the epicenter (transplant area) and the zones both proximal and distal to it. With the Image-Pro Plus 5.1 (Media Cybernetics) program, the intensity of green channel pixels corresponding to the Alexa 488 per area was quantified. A histogram, with previously calibrated intensity and spatial scale, was obtained from the intensity values contained within the image's bitmap to establish the intensity values such as the integrated intensity of each image. The optical density was determined in relation to the control group and expressed in pixels/mm^2.

2.3.3. Histology. The Kluver-Barrera staining method was performed on two specimens of each group; the sections were hydrated before being put into 95% alcohol; they were then placed in a Luxol Fast Blue solution overnight at 37°C. Following this, the excess colorant was removed using 95% alcohol and they were rinsed with distilled water; they were then washed with lithium carbonate and again rinsed with 70% alcohol and with distilled water. The sections were immediately placed in a purple Cresol solution for 6 minutes before being returned to the 70% alcohol and dehydrated with absolute alcohol. They were left to dry and fixed using Entellan synthetic resin (Merck). Finally, the samples were observed using a NIKON (Eclipse E600) microscope and the morphological changes were observed.

2.3.4. Electron Microscope. The specimens were fixed with a Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in a Sorensen solution) during 4 hours at 4°C. Following this, they were postfixed in 1% osmium tetroxide for 45 minutes at room temperature. The excess osmium tetroxide was removed by twice washing with distilled water for two minutes each time. They were dehydrated in alcohols of increasing concentration (50%, 70%, 95%, and 100%) to reach propylene oxide. Following dehydration, the tissue was included in Aldarite synthetic resin and was left to polymerize for 24 hours at 60 or 70°C. Once the blocks were carried out, semifine cuts were made to locate the zone to be evaluated before fine cuts were made that would be fixed to copper grids and stained with uranyl acetate and lead. The sections were analyzed with a Zeiss 906 transmission electron microscope and, for each group, 10 images were taken from the transition zone only between the PPN transplant and both the proximal and distal surrounding spinal cords in which only morphological changes were observed.

2.3.5. Statistical Analysis. The Graph Prism 5.0 statistics program was used for descriptive analysis; measures of central tendency and statistical dispersion were used alongside tables and graphs. For statistical inference, a nonparametric ANOVA analysis test was used with Kruskal-Wallis ranges to determine the differences between groups, followed by a Mann-Whitney U test to identify the groups between which there was a difference. $p < 0.05$ was considered significant.

3. Results

3.1. Motor Function Results. The hind extremity evaluation using the BBB rating scale showed severe motor function deterioration in the initial evaluation following the different experimental procedures. Scores improved marginally as time passed, especially for the PPN and BMSCs groups which reached an average rating close to 4 in contrast to the Control group which maintained a rating close to 1 (Figure 2).

3.2. Expression of GAP-43, Neuritin, and PBM: Microscopic Observations. In the qualitative evaluation of the histology images, a greater quantity of GAP-43 positive axons (Figure 3) and Neuritin was found in the transplant group compared to the Control group, in the zones both rostral and caudal to the injury (Figure 4). Furthermore, the PPN + BMSCs group axons were thicker than those observed in the PPN group (Figure 3). Finally, the presence of growth cones marked with Neuritin was only present in the PPN and PPN + BMSCs groups (Figure 4). A greater number of PBM-positive axons were also observed in the PPN and PPN + BMSCs groups in the zones rostral and caudal to the transplant (Figure 5).

3.3. Expression of GAP-43, Neuritin, and PBM: Quantification of Fluorescence Intensity. The GAP-43 fluorescence intensity was significantly greater in the groups that received a treatment (PPN + BMSCs, FG, and PPN) versus the Control group ($p < 0.05$) in both the rostral and caudal zones (Figures 6(a) and 6(b)); additionally, in the caudal zone (Figure 6(b)), the fluorescence intensity was significantly greater in the PPN + BMSCs and PPN groups versus FG ($p < 0.05$). The Neuritin fluorescence intensity (Figures 6(c) and 6(d)) and PBM (Figures 6(e) and 6(f)) in both the rostral and caudal zones was significantly greater in the PPN + BMSCs and PPN groups versus the FG and control groups ($p < 0.05$).

3.4. Electron Microscopy of Myelination. In the PPN and PPN + BMSCs groups, it was observed that the axon myelin

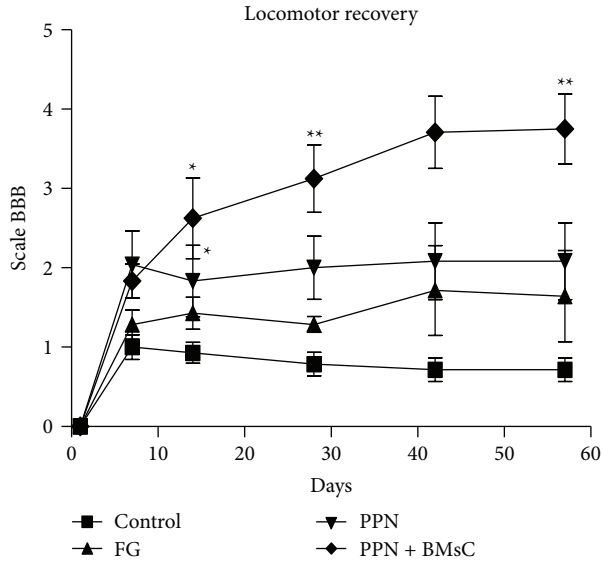


Figure 2: Analysis of locomotor function based on the BBB rating scale. y-axis corresponds to days of evaluation, where day 0 was the beginning of the evaluation within 24 hours after the transplant. The differences between the PPN and PPN + BMSCs groups can be observed when compared to the Control group (*) (Kruskal-Wallis test, $p < 0.05$); furthermore, upon comparing the treatment groups, only the PPN + BMSCs group presented a significant difference from day 30 onwards compared to FG (**) (Kruskal-Wallis test and Mann-Whitney U Test, $p < 0.05$ resp.), with no difference observed between the transplant groups. The graph represents the average ± standard error.

found had a well-defined and better-preserved structure; furthermore, there were a greater number of myelinated axons in contrast to Control and FG groups (Figure 7). Finally, in the PPN + BMSCs group, there were several thin axons rounded by a thin sheath of myelin and beside to a Schwann cell, which we consider the new axons (Figure 7). In both treatment groups, it was observed that the myelinated axons were ensheathed by the Schwann cells (Figure 7); on the other hand, in the PPN + BMSCs group, the BMSCs were found along with the axons and the Schwann cells (Figure 8).

3.5. Tissue Integration. From the samples analyzed with Luxol Fast Blue, it was observed that in the groups receiving PPN and PPN + BMSCs transplant, the surrounding spinal cord structure was adequately preserved and there was good acceptance between the transplanted PPN and the preserved SC (Figure 9).

4. Discussion

Functional recovery of the spinal cord following a traumatic injury with complete paralysis and secondary loss of sphincter control has been achieved using diverse therapeutic strategies in animal models. Nevertheless, therapeutic human trials carried out to date have had limited success. It is with that objective, therefore, that proposals for new alternatives using both single and combined treatment alternatives continue to be made.

It would appear that better results are achieved using combined treatments compared to single treatments. Using an acute model of complete transection, Guzen et al. [24] demonstrated that axonal regrowth and improved locomotor function took place following PPN transplantation; they also demonstrated that axonal regeneration and locomotor improvement increased when the PPN was combined with FGF-2, making it more effective than the use of PPN alone [24]. Using an acute model of complete transection, Koda et al. [25] also demonstrated that axonal regrowth and improved locomotor function occurred following the transplantation of BMSCs; however, the combination of BMSCs + BDNF showed greater effectiveness since it promoted a greater number of regenerated axons and increased locomotor function [25].

In this study, the therapy proposed to encourage axonal regrowth, remyelination, and functional recovery was the combined use of PPN and BMSC; considering that separately, each one of them has been shown to have a beneficial effect following the TSCI as previously mentioned.

The evaluation of axonal regrowth using the GAP-43 protein, known to be related to axonic fiber growth following a TSCI [26], showed a greater number of protein-positive fibers in the group transplanted with PPN than in Control group. Coinciding with Yuan et al. [27] in an axotomy model, GAP-43 positive fibers were found following the PPN transplant [27]. Following the PPN transplantation in an acute model of complete transection, Guzen et al. [24] also observed a greater quantity of GAP-43 positive fibers [24]. Furthermore, this study also observed that the PPN + BMSCs group had a greater number of GAP-43 positive fibers compared to the PPN group and they were also thicker than those of the PPN group. To date, no publications on the complete transection model are known to associate the use of BMSCs and the expression of GAP-43. However, Čížková et al. [28] observed the expression of GAP-43 following the transplantation of BMSCs in a compression model [28]. Finding GAP-43 positive fibers in another study using a contusion model, Kamada et al. [29] demonstrated that axonal regrowth was promoted following the transplantation of BMSCs [29]. The expression of GAP-43 might be mainly due to the PPN since it expresses different substances to support its regeneration such as trophic factors, adhesion molecules, and extracellular matrix molecules; [30] these factors provide a stimulating environment to facilitate the growth of CNS axons and support locomotor function [30]. However, since this expression is greater in the combined group, this could also be due to the contribution of the BMSCs given that the use of BMSCs in TSCI helps modulate the CNS environment to promote self-repair, secreting trophic substances that promote axonal growth [14].

As well as observing the presence of GAP-43, this study only found Neuritin immunoreactivity in the transplant groups PPN and PPN + BMSCs, indicative of the presence of growth cones; however, there was no significant difference between the transplant groups. Neuritin is the protein implicated in neuronal plasticity, relating to neurite

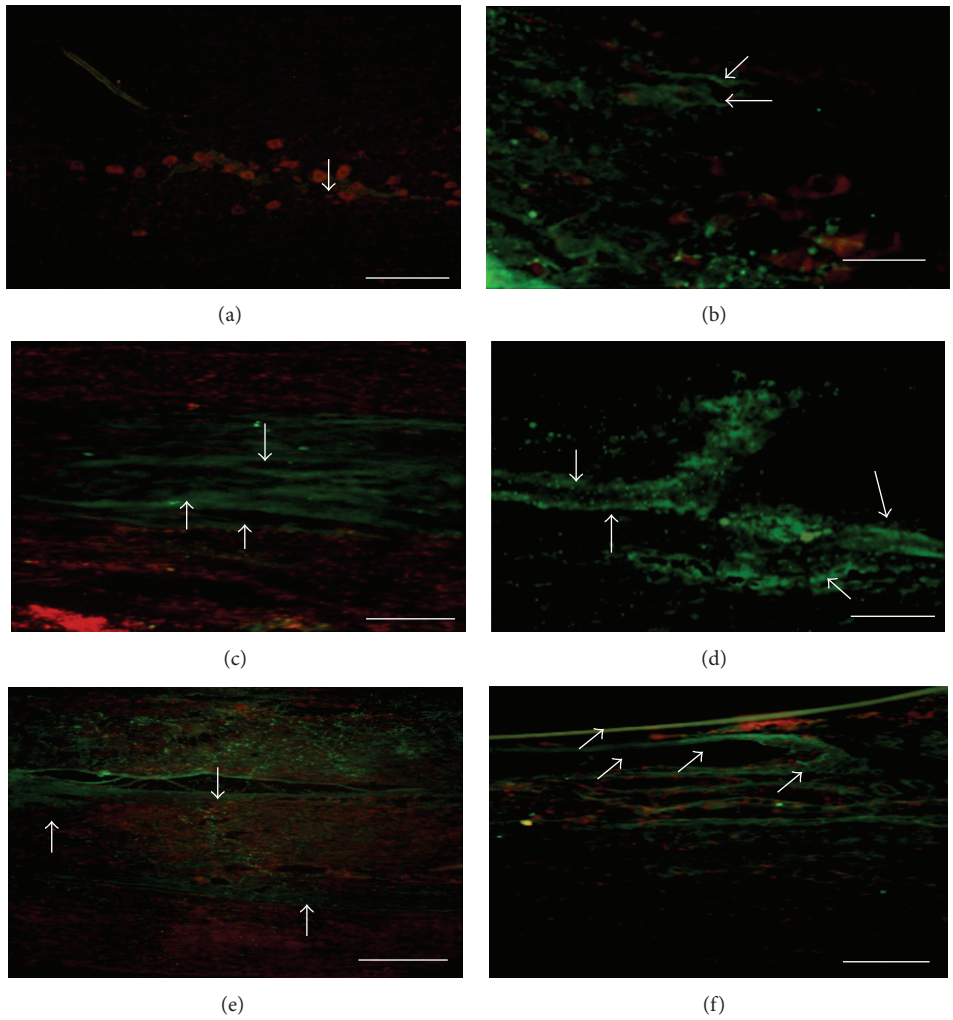


Figure 3: Photomicrography of the expression of GAP-43 in spinal cord. (a) Control group. (b) Fibrin glue group (FG). (c) PPN group, rostral zone of the SC. (d) PPN + BMSCs group, rostral zone of the SC. (e) PPN group, caudal zone of the SC. (f) PPN + BMSCs group, caudal zone of the SC. A greater quantity of GAP-43 positive fibers can be observed in panels (c) to (f) (white arrows, Alexa 488, green color), compared to panels (a) and (b). Furthermore, and easily visible, the positive fibers which were found in the PPN + BMSCs group ((e) and (f)) are thicker than those ones which were in the PPN group ((c) and (d)). All the tissue was contrasted with propidium iodide (nuclei red color). Calibration bar 20 μm.

growth [31]. There are no publications on complete section models in respect to this marker; however, Sarah Busch and colleagues have associated the presence of growth cones with the regrowth of sensory axons in a spinal cord compression model [32].

Myelination is essential to maintaining optimum function of the CNS since it promotes the conduction of nerve impulses and provides metabolic and trophic support; [33] so, when a TSCI occurs, the destruction of myelin affects motor and sensory function in the aforementioned manner. Structural affectation in different TSCI models can be identified by evaluating the level of myelination. PBM has been used to evaluate the level of myelination in different experimental models. In this study, a greater expression of PBM was observed in the transplant groups, especially in the distal segment of the spinal cord and mainly in the PPN + BMSCs group. Furthermore, in the electron microscope study, it was demonstrated that the PPN + BMSCs group had a greater quantity of myelinated axons which were more robust and which had ramifications. There are no studies known to evaluate the effect of PPN and BMSCs transplant on PBM in a chronic complete transection model. However, in an acute complete transection model, Chen et al. [34] demonstrated that they obtained a greater number of myelinated axons after transplanting BMSCs and the myelin had a uniform and more dense structure [34]. This property can be explained by the capability of the BMSCs to differentiate into myelinating cells. This is in line with the studies carried out by Akiyama et al. [35] who used a model of radiation injury, in which the myelinating cells were destroyed after transplantation of BMSCs, demonstrating that they promoted the myelination of bare axons which improved the nerve impulse [35].

Additionally, cells with a morphology that was different from that of the nervous tissue were observed near the

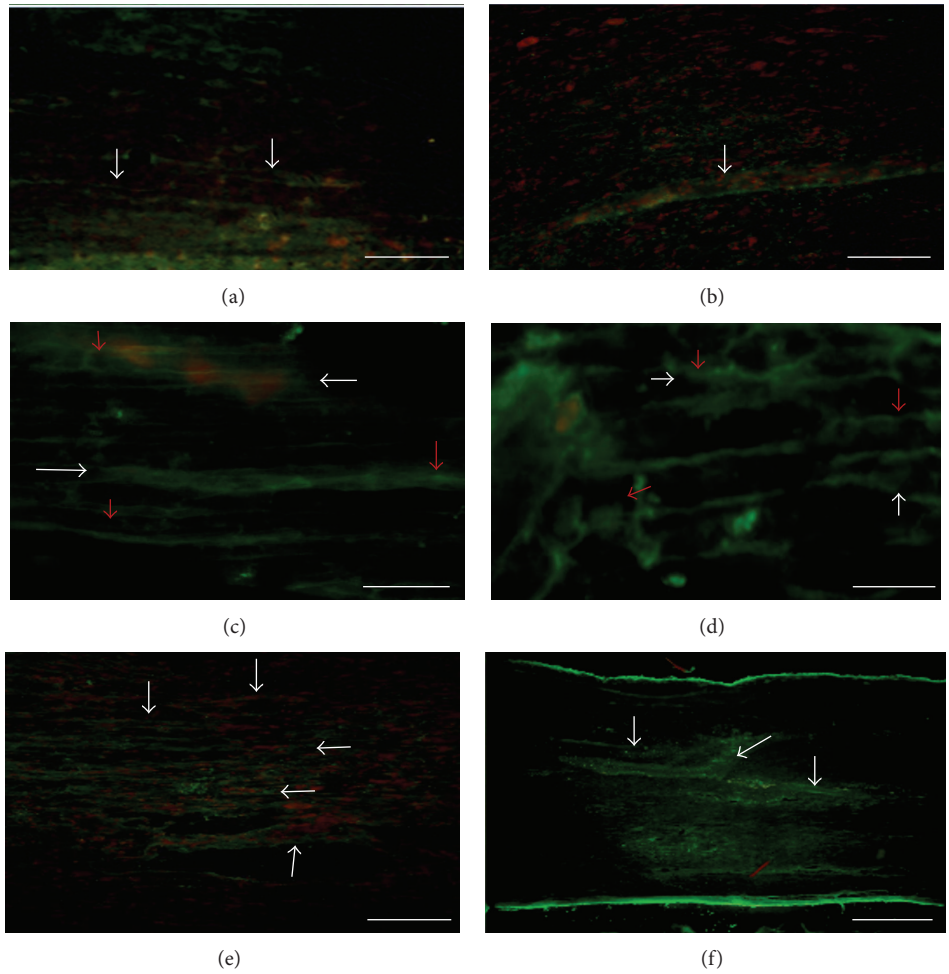


Figure 4: Photomicrography of the expression of Neuritin in spinal cord. (a) Control group. (b) Fibrin glue group (FG). (c) PPN group, rostral zone of the SC. (d) PPN + BMSCs group, rostral zone of the SC. (e) PPN group, caudal zone of the SC. (f) PPN + BMSCs group, caudal zone of the SC. A greater quantity of Neuritin positive fibers can be observed in panels (c) to (f) (white arrows, Alexa 488, green color), compared to panels (a) and (b). We can also observe that growth cones (red arrows) were only presented in the treated groups (panels (c) and (d)). All the tissue was contrasted with propidium iodide (nuclei red color). Calibration bar 20 μm.

myelinated axons; these cells could correspond to differentiated BMSCs, which can differentiate into myelinating cells as mentioned above [35], and, together with the Schwann cells resulting from the PPN, they help bring about a better myelination of regenerated axons as was demonstrated by Dam-Hieu et al. [36] in a model of hemicordotomy, in which the axons were myelinated by the actions of the Schwann cells following the transplantation of PPN [36].

Finally, and as a result of the beneficial mechanisms already mentioned in relation to both the PPN and the BMSCs, it was observed that, in the PPN and PPN + BMSCs transplant groups, the surrounding spinal cord structure was adequately preserved and that there was good acceptance with the PPN, which can be categorized as neuroprotection. This is in line with the study published by Guizar-sahagun et al. [3], in which they observed that the use of PPN acted like a shock absorber for substances produced by the secondary injury mechanisms, protecting the SC surrounding the injury zone, expressed as improved medullary tissue preservation [3]. On the other hand, Feng et al. [30] showed that, following the use of PPN in a model of contusion, there was greater neuronal preservation, which is due to the fact that the PPN promotes a microenvironment in which the Schwann cells secrete growth factors such as BDNF, NGF, and NT3 which improve neuronal survival [30]. It has been seen that the neuroprotective capacity of the BMSCs comes from the secretion of different substances, which can promote immunomodulation, repair, remyelination, axonal regrowth, and improved function. Using a model of compression, Quertainmont et al. [14] demonstrated that, in transplanting BMSCs, their neuroprotective effects came from the secretion of molecules such as the ciliary neurotrophic factor (CNT-F), the monocyte chemoattracting protein-1 (MCP-1), and the granulocyte-macrophage colony-stimulating factor (GM-CSF) which support the survival and differentiation of oligodendrocyte precursor cells, promoting the clearance of myelin debris and protecting the neurons and glial cells from apoptosis. They also observed an increase in the secretion

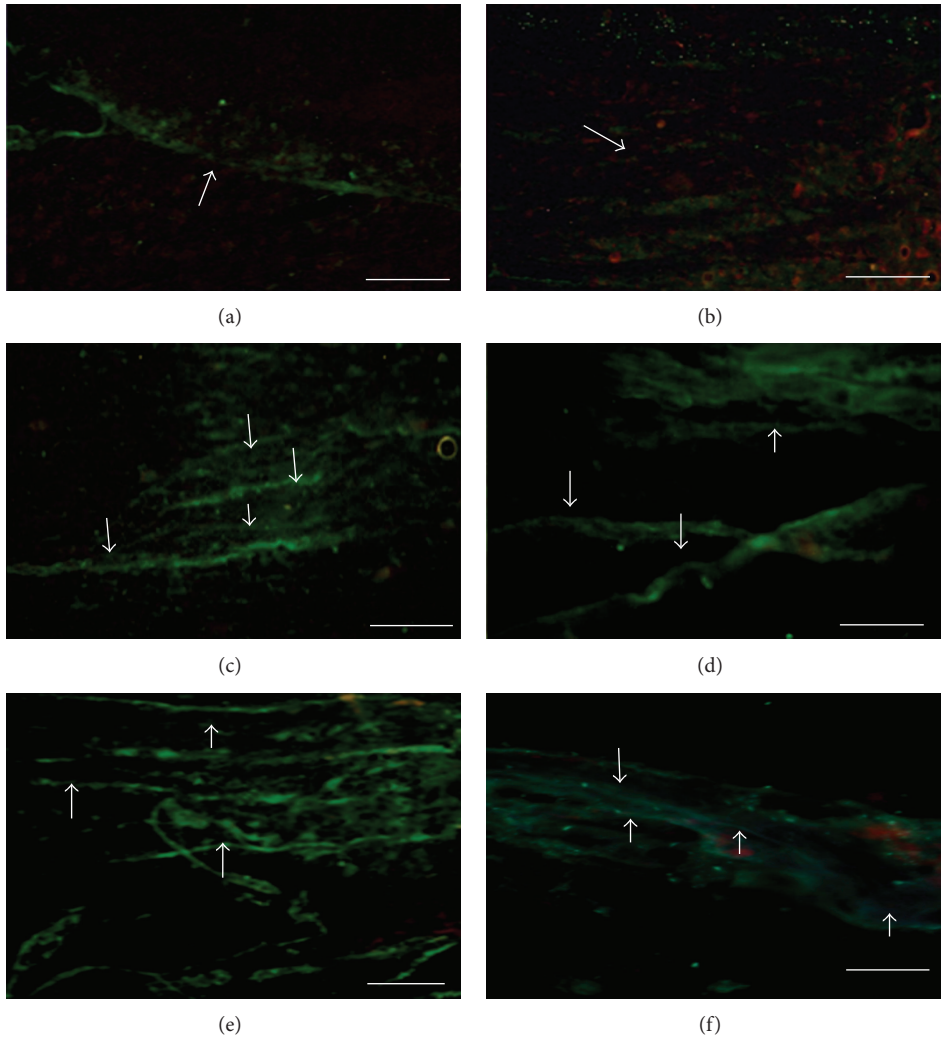


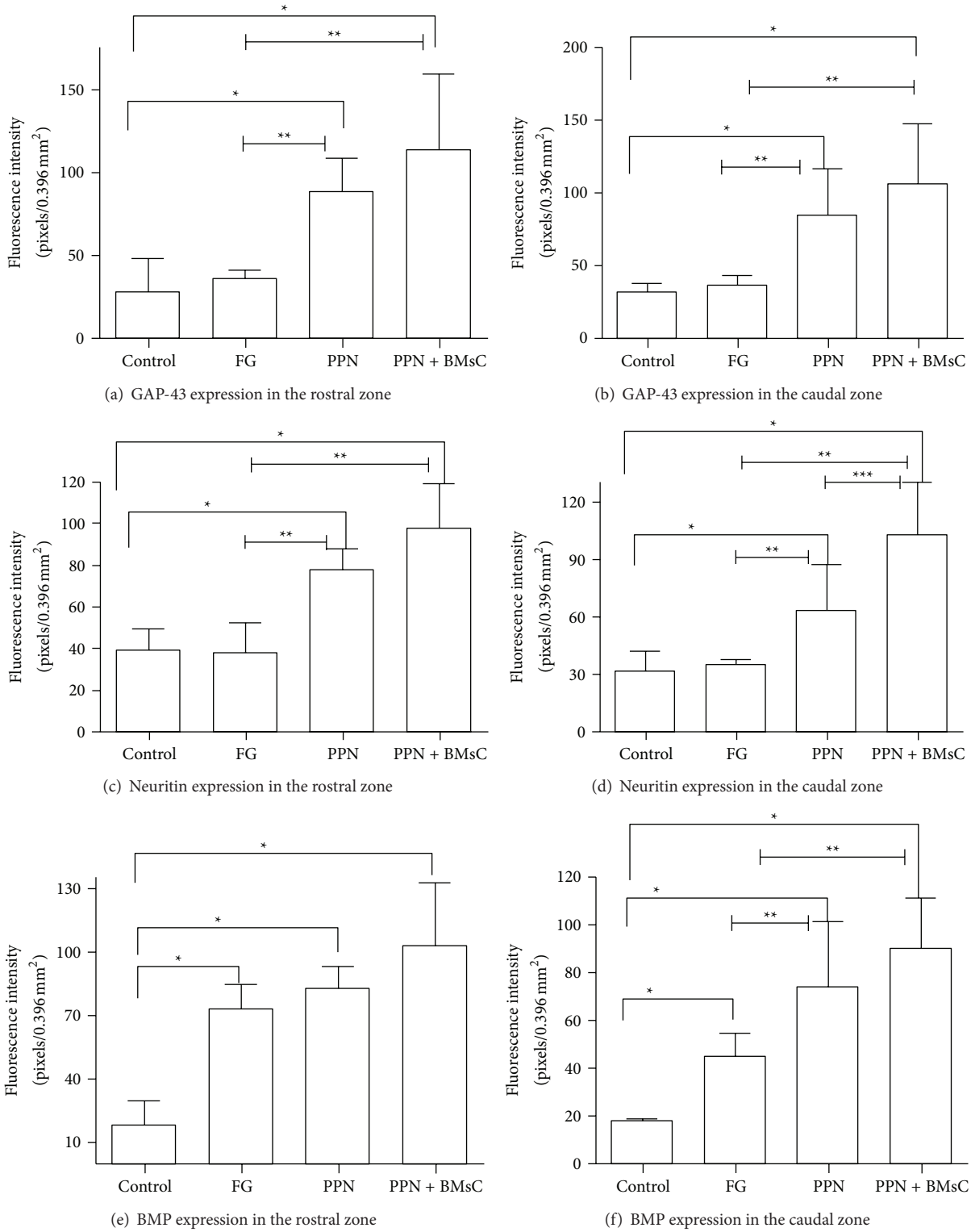
Figure 5: Photomicrography of the expression of Basic Protein Myelin in spinal cord. (a) Control group. (b) Fibrin glue group (FG). (c) PPN group, rostral zone of the SC. (d) PPN + BMSCs group, rostral zone of the SC. (e) PPN group, caudal zone of the SC. (f) PPN + BMSCs group, caudal zone of the SC. A greater quantity of PBM-positive fibers can be observed in panels (c) to (f) (white arrows, Alexa 488, green color) in contrast to panels (a) and (b). All the tissue was contrasted with propidium iodide (nuclei red color). Calibration bar 20 μm.

of anti-inflammatory cytokines such as IFN-γ and IL-10 as well as the secretion of growth factors such as BDNF and NGF, which help protect the neurons during toxic events, promote regrowth, and repair and reorganize the neuronal connections and which also stimulate neurogenesis and protect tissue, decreasing scar formation. Also observed were the antiangiogenic effects of the secretion of the vascular endothelial growth factor (VEGF) [14].

Despite the fact that there were significant locomotion differences between transplanted and nontransplanted animals, the improvement was modest. On the other hand, although there was a tendency in favor of combined transplantation (PPN + BMSCs), the difference versus PPN group was not significant. In both cases, it can be due to the short term functional follow-up, because it has been seen that improvement begins at the third month after implemented treatment. Poor functional improvement was observed in models of complete transection that received no additional treatment, obtaining an average BBB rating scale score of 4 points [37]. Following transplantation of PPN in an acute model of complete transection, Guzen et al. [24] obtained an average score of 5 on the BBB rating scale eight weeks after treatment [24]. Following the transplantation of BMSCs in another acute injury study, Chen et al. [34] observed an average of 8 points on the same scale following eight weeks of treatment [34]. It is possible that there is additional functional improvement with long term follow-up as reported by Vaquero and Zurita in a severe contusion model in which the experimental animals achieved a 13 point score with BMSCs transplantation after a one-year follow-up [21].

5. Conclusions

The transplant of PPN + BMSCs in a chronic complete spinal cord transection model showed significantly great myelination in the preserved spinal cord rostral and caudal to the



(a) GAP-43 expression in the rostral zone

(b) GAP-43 expression in the caudal zone

(c) Neuritin expression in the rostral zone

(d) Neuritin expression in the caudal zone

(e) BMP expression in the rostral zone

(f) BMP expression in the caudal zone

Figure 6: Fluorescence intensity analysis. Panels (a) and (b), expression of GAP-43: in both areas of the SC, a significant difference can be observed in the FG, PPN, and PPN + BMSCs groups compared to the Control group (Kruskal-Wallis test, $p < 0.05$) as well as a significant difference in the treatment groups compared to the FG group (Mann-Whitney U test, $p < 0.05$). Panels (c) and (d), expression of Neuritin: it can be observed that the treatment groups obtained a significant difference when they are compared to the Control and FG groups (Kruskal-Wallis test and Mann-Whitney U test, $p < 0.05$). Panels (e) and (f), expression of PBM in the SC: in both areas, a significant difference is observed between the treated groups and the Control and FG groups (Kruskal-Wallis test and Mann-Whitney U test, $p < 0.05$); furthermore, in the distal zone, a significant difference can be observed between the PPN + BMSCs group compared with the PPN group (Mann-Whitney U test, $p < 0.05$). * is the difference with Kruskal-Wallis $p < 0.05$, and ** is the difference with U-MANN $p < 0.05$.

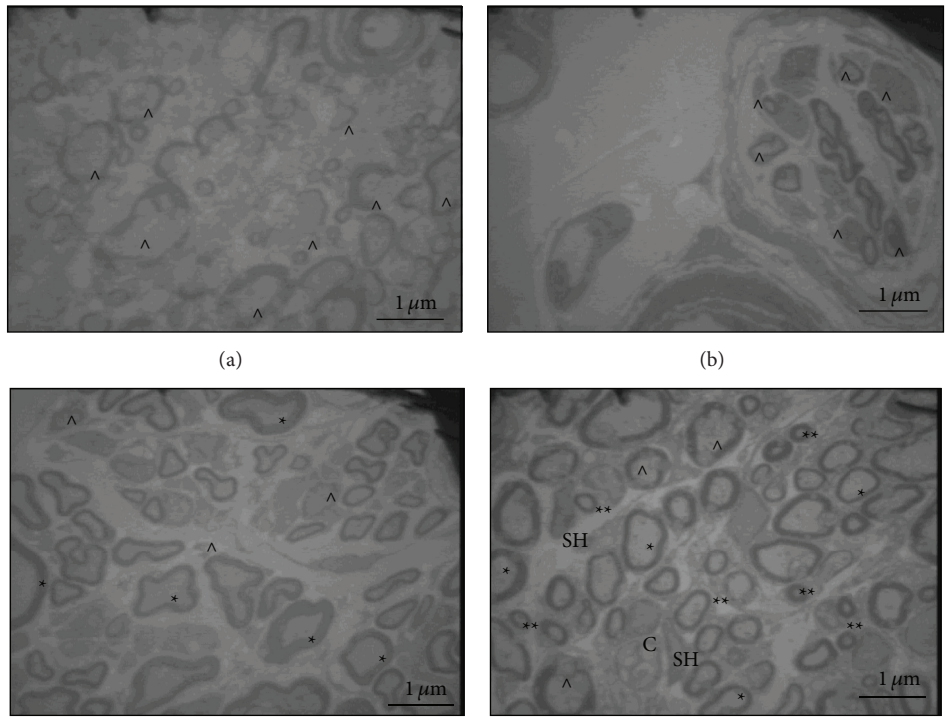


Figure 7: Electron photomicrography of distal stump to spinal cord. (a) Control; demyelinated axons and axons in process of demyelination (^) (1600x). (b) FG; damaged axons (^) (1600x). (c) PPN; myelinated axons (*), macrophage close to a damage axon (MA), and damaged axons (^) (1600x). (d) PPN + BMSCs myelinated axons (*), newly formed axons (**), some damaged axons (^), and Schwann cell (SH) beside its axon and BMSCs (C) beside a Schwann cell (SH) (1600x). The images were taken with a transmission electron microscope LEO 906 E. Calibration bar 1 μm.

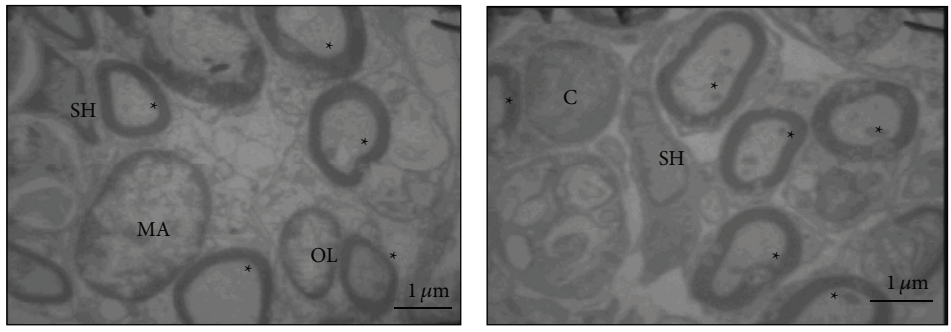


Figure 8: Electron photomicrography of distal stump to spinal cord. (a) PPN; Schwann cell (SH) interacts with a myelinated axon (*); an oligodendrocyte (OL) can also be observed beside an axon, and a macrophage (MA) is seen in the left corner (1600x); (b) BMSC interacts (C) with a Schwann cell (SH) beside a myelinated axon (*) (4646x). The images were taken with a transmission electron microscope LEO 906 E. Calibration bar 1 μm.

transplant area compared to PPN. However, although axonal regrowth and functionality were not significant, the PPN + BMSCs group showed high levels. The transplanted groups showed significantly greater axonal regrowth, myelination, and functionality than the Control groups. Additional studies are needed to evaluate the long term functional effect. The use of new combinations that potentially increase locomotor function in the same injury model used in this study is also proposed.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

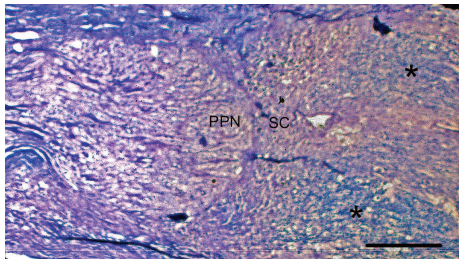


Figure 9: Spinal cord transplant interface. Photomicrography of a specimen from the PPN + BMSCs group in which excellent adhesion between the transplanted nerve (PPN) and the distal spinal cord (SC) stump can be observed. The spinal cord shows a good level of myelination (*). Kluver-Barrera staining. Calibration bar 500 μm.

Acknowledgments

Special thanks are to Doctor Karina Chávez Rueda of the Centro Médico Nacional Siglo XXI Pediatric Hospital for carrying out the flow cytometry studies. To carry out the study, finance was received from the Health Research Promotion Fund of the Mexican Institute of Social Security FOFOI/FIS no. 2005-1/I/036. Vinnitsa Buzoianu-Anguiano received a grant from the National Council for Science and Technology (CONACYT) and from the Mexican Social Security Institute (IMSS).

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 389531, 9 pages
http://dx.doi.org/10.1155/2015/389531

Clinical Study

Cortical Reorganization Is Associated with Surgical Decompression of Cervical Spondylotic Myelopathy

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Received 3 December 2014; Revised 25 May 2015; Accepted 3 June 2015

Academic Editor: Brandon A. Miller

Background. Cervical spondylotic myelopathy (CSM) results in sensorimotor limb deficits, bladder, and bowel dysfunction, but mechanisms underlying motor plasticity changes before and after surgery are unclear. *Methods*. We studied 24 patients who underwent decompression surgery and 15 healthy controls. Patients with mixed upper and lower limb dysfunction (Group A) and only lower limb dysfunction (Group B) were then analysed separately. *Results*. The sum amplitude of motor evoked potentials sMEP ($p < 0.01$) and number of focal points where MEPs were elicited (N) ($p < 0.001$) were significantly larger in CSM patients compared with controls. For Group A (16 patients), sMEP ($p < 0.01$) and N ($p < 0.001$) showed similar findings. However, for Group B (8 patients), only N ($p = 0.03$) was significantly larger in patients than controls. Group A had significantly increased grip strength ($p = 0.02$) and reduced sMEP ($p = 0.001$) and N ($p = 0.003$) after surgery. Changes in sMEP (cMEP) significantly correlated inversely with improved feeding ($p = 0.03$) and stacking ($p = 0.04$) times as was the change in number of focal points (NDiff) with improved writing times ($p = 0.03$). Group B did not show significant reduction in sMEP or N after surgery, or significant correlation of cMEP or NDiff with all hand function tests. No significant differences in H reflex parameters obtained from the flexor carpi radialis, or central motor conduction time changes, were noted after surgery. *Discussion*. Compensatory expansion of motor cortical representation occurs largely at cortical rather than spinal levels, with a tendency to normalization after surgery. These mirrored improvements in relevant tasks requiring utilization of intrinsic hand muscles.

1. Introduction

Cervical spondylotic myelopathy (CSM) is one of the most common causes of spinal cord dysfunction in older individuals [1–3]. CSM is a chronic and progressive disease resulting from degenerative changes in the spine that gives rise to cord and nerve root impingement by osteocartilaginous elements [4]. These lesions cause much morbidity in patients, including sensorimotor limb deficits, bladder, and bowel dysfunction. Many patients with CSM are treated surgically with the hope of preventing further neurological deterioration or achieving some functional recovery [4, 5]. However, the physiological mechanisms underlying the recovery of motor function after CSM surgery are poorly understood.

Evidence in the medical literature suggests that the improvement of motor function after surgical decompression in CSM patients may occur via synaptic changes and dendritic sprouting in the cortical and spinal cord neuron pools [6, 7]. Firstly, the natural process of functional recovery without medical intervention in many pathological

situations involves plasticity changes in the motor cortex. For example, transcranial magnetic stimulation (TMS) studies in stroke patients have shown that motor recovery is associated with improved corticospinal conduction as well as cortical reorganization [8, 9]. This recovery process is not limited to the event of cortical damage. In fact, Nishimura et al. [10] have demonstrated that functional reorganization in bilateral premotor and primary motor areas took place after lateral corticospinal tract transection at the cervical level in macaque monkeys. These plasticity changes in the motor cortices were associated with restoration of skilled finger movements. Similarly, neuroimaging studies in humans have affirmed that rapid cortical and subcortical reorganization are a common occurrence after spinal cord injury and/or myelitis [11–13]. In patients with cervical myelitis, robust changes within the sensorimotor cortex were inversely correlated with the severity of the spinal cord damage [11]. Taken together, these findings strongly indicate the importance of cortical reorganization in sensorimotor function improvement after spinal cord injury.

Without medical intervention, the natural recovery process following spinal cord compression is slow and largely depends on the extent of the injury sustained [12, 14, 15]. A number of trials have shown that CSM patients treated with decompression surgery experienced neurological improvements and, as such, surgical intervention is often recommended in moderate and severe CSM cases [4, 13]. In addition, serial functional magnetic resonance imaging (fMRI) studies have captured the evolving changes in the cerebral cortex in CSM patients following surgical decompression [7, 16]. However, no study thus far has compellingly shown the direct relationship between cortical plasticity and the degree of motor improvement after spinal cord injury.

An emerging modality used to study functional organization in the human motor cortex is TMS [2, 8, 17]. It is a noninvasive tool that measures conduction in the descending corticospinal pathways and is capable of rapidly evaluating output assessing the functional organization and reorganization of the human motor cortex [8, 18, 19]. Within this framework, we aim to investigate the association between cortical reorganization and motor function improvement after cervical decompression surgery. We hypothesize that a correlation exists between the plasticity in the cortex and improvement in motor function scores (as measured by the Modified Japanese Orthopaedic Association Score) [2, 17] and detailed tests of hand function [20] in moderate-to-severe CSM patients four months after spinal cord decompression surgery. Secondarily, we investigate compensatory motor cortex representation changes in CSM patients in relation to healthy controls.

2. Methods

2.1. Subjects. With ethics committee (Singapore General Hospital ethics committee) approval, patients presenting with clinical features of CSM of at least 6 months' duration who were listed for spinal cord decompression surgery were recruited with informed consent obtained. We excluded patients with suspected traumatic spinal injury, or any underlying medical or neurological condition which may confound electrophysiological findings. MRI of the cervical spine was performed in all patients within 1 month before surgery. No physiotherapy sessions were scheduled for these patients after surgery. Every recruited patient underwent TMS and motor function testing 1 month prior to and 4 months after surgery. The operation is usually anterior laminectomy of the cervical spine, or any additional procedure stabilization. We also recruited healthy controls for comparison.

2.2. Transcranial Magnetic Stimulation. TMS mapping of the left hemisphere was performed using a Medtronic (Medtronic Corporation, USA) figure-of-eight-shaped C-B60 coil with 7 cm internal diameter connected to a Medtronic R8 unit generating a peak magnetic field of 2.2 Tesla. The coil was placed tangentially over the skull with the handle pointing backwards and perpendicular to the direction of the central sulcus at approximately 45 degrees to the midline to evoke an anteromedially directed current in the brain.

The vertex, designated as intersection of the interaural line and the nasion-inion connection, was used as an anatomical landmark for finding the optimal position (hotspot) for eliciting motor-evoked potentials (MEPs) from the right first dorsal interosseous (FDI). This is defined as the position with the lowest stimulation intensity needed to elicit an MEP. At the hotspot, the resting motor threshold (rMT) is determined as the position where the lowest TMS intensity will elicit an MEP at a vertical gain of 50 μV/division for 5 out of 10 stimulations. Once these procedures were completed, the hotspot is placed as the centre of a square-shaped 25-position grid drawn along both the anteroposterior and the mediolateral axes on the subject's head. Each point is spaced 1 cm apart from its adjacent position. The map for the right FDI was then obtained by stimulating each point of the grid lying over the motor strip. For each scalp position, we recorded the mean of MEP amplitudes evoked by 5 stimulations at 110% of the rMT. During the recording, which required EMG silence, muscular activity was constantly monitored. MEPs were amplified, filtered, and recorded on a Medtronic Keypoint electromyography machine with a band pass of 20 to 2000 Hz for analysis. Continuous EMG and sound monitoring ensured only nonfacilitated responses will be included for analysis.

TMS parameters obtained were the sum amplitude of MEPs (sMEP) of the entire 25-point grid and number of positions (N) where MEPs could be elicited. We also computed the difference in sMEP (cMEP) and N (NDiff) before and after surgery in each patient. For comparison, healthy age-matched controls had similar TMS motor mapping performed.

To better ascertain if corticospinal excitability changes occur at the spinal or supraspinal levels, H reflexes were obtained from the right flexor carpi radialis by stimulating the median nerve at the elbow level. Both H amplitude and H/M ratios were noted, where M referred to amplitude of the flexor carpi radialis compound muscle action potential, as described previously [21, 22].

Central motor conduction times (CMCT) were also obtained from both upper and lower limbs in all patients before and after surgery. CMCT methodology was in accordance with previously published studies by the same authors [21, 22].

2.3. Motor Function Testing. Apart from clinical history and physical examination, each patient's motor function was quantitatively assessed using Modified Japanese Orthopaedic Association Score Scale (mJOAS) [17, 23] and Jebsen test of hand functions (JHFT) [20]. The tests were done at baseline and 4 months after operation and documentation was by an investigator who did not perform the surgical procedure.

2.4. Data Analysis. As CSM can result in exclusively upper limb or lower limb complaints as well as mixed upper and lower limb features, we separated patients into two groups. In Group A, all had mixed upper and lower limbs features, but patients in Group B had features exclusive to the lower limbs, in line with the mJOAS described above. None of the patients experienced sphincter disturbances.

Statistical calculations were made using SPSS for Windows software. The Wilcoxon Signed-Rank test was used to compare means and Spearman correlation coefficient was employed to examine the relation between MEP characteristics and functional changes in patients after surgery. A p value of <0.05 denoted statistical significance.

3. Results

All 24 patients (16 males, 8 females, mean age ± SD: 58.2 ± 11.5) were right handed as were the 15 healthy age-matched control subjects.

mJOA scores were significantly improved after surgery for all patients ($p = 0.03$).

For all patients, we found that sMEP ($p = 0.0014$) and N ($p = 0.0008$) were significantly larger preoperatively.

The sMEP ($p = 0.012$) and N ($p = 0.0008$) were significantly larger in preoperative CSM patients compared with healthy controls.

Separately, for Group A (16 patients), sMEP ($p = 0.003$) and N ($p = 0.001$) were also larger than healthy controls. However, for Group B (8 patients), only N ($p = 0.0026$) was significantly larger than healthy controls.

Postoperatively, no significant differences in sMEP for Group A ($p = 0.08$) or Group B ($p = 0.796$) were found compared with controls. However, for N, Group A ($p = 0.01$) was still significantly larger than healthy controls. This was not seen in Group B ($p = 0.12$) compared with healthy controls.

For Group A, we found significantly reduced sMEP ($p = 0.001$) and N ($p = 0.003$) after surgery. In addition, significantly increased grip strength ($p = 0.02$) and improved time for picking small objects ($p = 0.04$) were noted. Specifically, cMEP, in terms of reduction of sum of MEP amplitudes after surgery, significantly correlated with improved feeding ($r = 0.25$, $p = 0.03$) and stacking ($r = 0.52$, $p = 0.04$) times. NDiff in terms of reduction after surgery in number of excitable positions where MEPs were elicited significantly correlated with improved writing times ($r = 0.48$, $p = 0.03$).

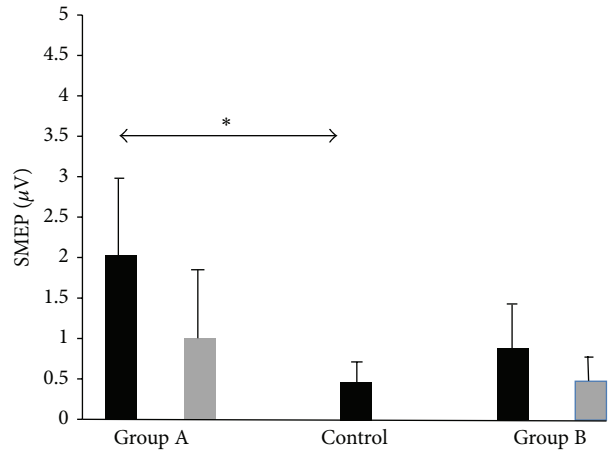

Figure 1: sMEP findings graphically. Asterisks denote statistical significance. Preoperative bars are black and postoperative bars are grey. sMEP, sum of MEP amplitudes in mV in vertical axis. Horizontal axis depicts patient and control groups.

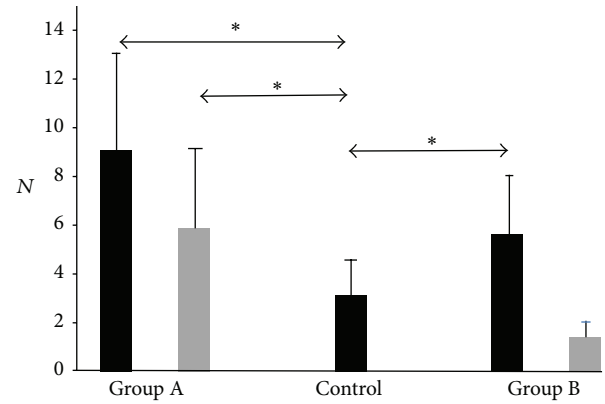

Figure 2: N findings graphically. Asterisks denote statistical significance. Preoperative bars are black and postoperative bars are grey. Vertical axis depicts number of excitable positions where MEP is elicited (N). Horizontal axis depicts patient and control groups.

For Group B, there was no significant reduction in sMEP or N after surgery, and no significant correlation was found for cMEP or NDiff with all hand function tests.

We did not find significant differences in CMCT from all 4 limbs and H reflex parameters before and after surgery.

Table 1 summarizes study results of patients and controls.

Figures 1 (sMEP) and 2 (N) depict MEP mapping findings graphically. Asterisks denote statistical significance. Preoperative bars are black and postoperative bars are grey.

Figure 3 is a schematic diagram depicting motor output mapping of a patient in Group A preoperatively and postoperatively.

4. Discussion

In the first TMS study of this nature to our knowledge, we sought to provide a vital connection between existing

Table 1: Summary of experimental results in all patients.

	Preoperative		Postoperative		Significance
mJOA	12.7 (2.81)		13.81 (3.1)		$p = 0.03^*$
sMEP	1.64 (1.88)		0.82 (0.89)		$p = 0.0014^*$
N	7.86 (3.93)		5.22 (2.58)		$p = 0.0008^*$
Group	A	B	A	B	
sMEP	2.03 (1.54)	0.89 (0.55)	1.01 (1.05)	0.48 (0.31)	Group A ($p = 0.003$)* Group B ($p = 0.65$)
N	9.07 (4.00)	5.60 (2.80)	5.87 (2.95)	4.00 (1.41)	Group A ($p = 0.0001$)* Group B ($p = 0.0026$)*
Jebsen tests					
Write	23.98 (25.49)	12.11 (5.84)	20.97 (20.08)	11.71 (6.10)	Group A ($p = 0.60$) Group B ($p = 0.25$)
Turn page	9.56 (9.15)	5.98 (2.22)	7.19 (3.53)	7.89 (4.86)	Group A ($p = 0.17$) Group B ($p = 0.18$)
Lift small object	11.43 (7.06)	9.65 (4.26)	9.19 (4.13)	9.54 (6.10)	Group A ($p = 0.04$)* Group B ($p = 0.98$)
Feed	13.20 (6.74)	13.06 (7.12)	12.03 (5.89)	11.38 (5.74)	Group A ($p = 0.58$) Group B ($p = 0.12$)
Stack	5.64 (6.43)	2.85 (2.17)	4.00 (6.13)	2.78 (1.07)	Group A ($p = 0.31$) Group B ($p = 0.83$)
Lift light can	4.23 (3.88)	4.69 (2.87)	4.21 (3.91)	4.56 (3.90)	Group A ($p = 0.43$) Group B ($p = 0.65$)
Lift heavy can	5.18 (2.34)	5.14 (3.34)	4.87 (2.02)	4.89 (3.33)	Group A ($p = 0.34$) Group B ($p = 0.62$)
Other tests					
9-hole peg	68.36 (41.78)	52.63 (30.00)	57.39 (28.63)	59.77 (34.12)	Group A ($p = 0.26$) Group B ($p = 0.14$)
Tap	68.63 (12.09)	70.43 (10.83)	69.79 (4.57)	74.79 (8.13)	Group A ($p = 0.92$) Group B ($p = 0.14$)
Pinch grip strength	15.55 (7.46)	20.86 (3.8)	17.87 (7.33)	22.64 (4.68)	Group A ($p = 0.02$)* Group B ($p = 014$)
Electrophysiology					
CMCT					
R UL	10.77 (3.22)	7.89 (2.34)	9.88 (2.16)	7.69 (2.87)	Group A ($p = 0.23$) Group B ($p = 0.31$)
L UL	11.65 (3.45)	7.99 (2.77)	10.45 (2.98)	7.62 (2.96)	Group A ($p = 0.32$) Group B ($p = 0.45$)
R LL	18.34 (3.98)	19.23 (4.11)	17.97 (4.06)	19.86 (4.68)	Group A ($p = 0.64$) Group B ($p = 0.46$)
L LL	19.11 (4.07)	19.25 (4.87)	18.78 (4.39)	20.12 (4.61)	Group A ($p = 0.51$) Group B ($p = 0.48$)
H amplitude	1.22 (0.53)	1.34 (0.45)	1.21 (0.47)	1.29 (0.51)	Group A ($p = 0.51$) Group B ($p = 0.48$)
H/*M*	0.82 (0.23)	0.91 (0.22)	0.77 (0.36)	0.86 (0.28)	Group A ($p = 0.41$) Group B ($p = 0.57$)

Mean values are indicated (standard deviation).
All hand function test results are in seconds.
In healthy controls, mean sMEP was 0.51 (0.28) and *N* was 3.36 (1.21).
CMCT: central motor conduction time (m/s); R: right; L: left; UL: upper limb; and LL: lower limb.
H/*M*: *H* amplitude (mV)/*M* amplitude (mV).
∗ denotes statistical significance at $p < 0.05$.

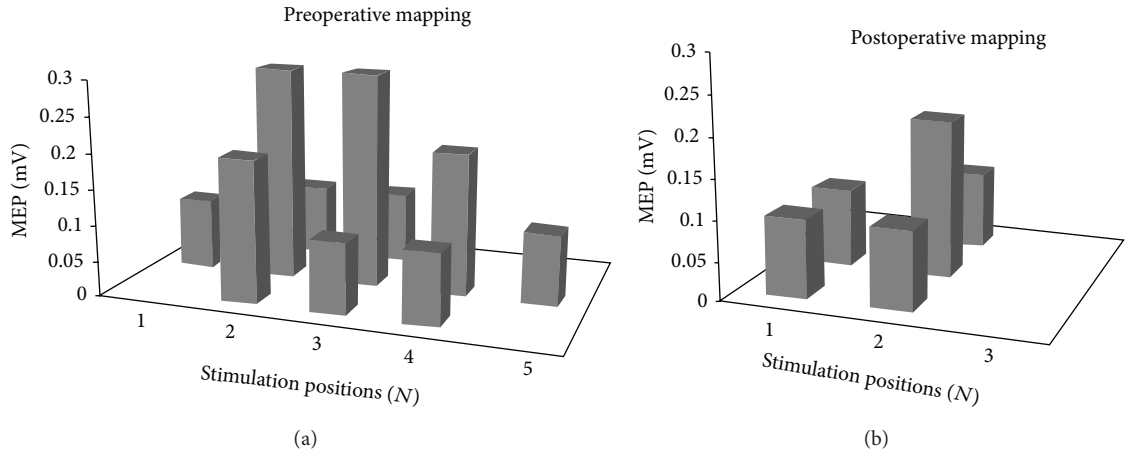

Figure 3: Schematic diagram depicting motor output mapping of a patient in Group A. In the preoperative grid, sMEP is 1.7 mV as sum total of 10 stimulation positions eliciting an MEP ($N = 10$). Postoperatively, sMEP was reduced to 0.7 mV and N to 5. sMEP, sum of MEP amplitudes in mV.

studies using functional imaging and the recovery process after decompression surgery in CSM.

Early imaging studies in CSM have focused on morphological changes in operated CSM patients. Fukushima et al. [24] showed that good functional outcome after surgery is correlated with a minimum re-expanded cord area in 55 patients. Baba et al. [13] separately studied 56 patients and concluded that early postoperative cord expansion reflected improved clinical status and suggested that this may be due to enhanced "intracord plasticity." However, these studies did not utilize electrophysiology as a bridge to explain clinical and morphological changes.

The advent of functional imaging, including PET and fMRI, provided new information on brain remodelling by virtue of blood flow changes. In terms of spinal cord lesions, traumatic spinal cord injury (SCI) is known to induce expanded brain activation towards the leg areas, thalamus, and cerebellum as seen in PET studies [25]. In a separate group of 6 SCI patients, fMRI showed initial decrease and then increase of activation of sensorimotor areas [26], reflecting the dynamic response of brain function probably as a compensatory mechanism. In terms of morphology, complete SCI patients exhibited reduced gray matter volume in the primary motor, medial prefrontal, cingulate, and cerebellar cortex, in addition to diffusion tensor imaging (DTI) changes in cortical [27, 28] and brainstem motor areas [29]. When interpreting these findings, it should be noted that SCI may differ in onset, chronicity, and extent, which may in turn affect the cortical or subcortical changes observed.

Specifically for CSM, few studies have been published to date addressing fMRI changes before and after decompression. Holly et al. [16] found evidence of expanded cortical representation of the affected arm. Following surgery, distinct reorganization of this representation was seen but not in any consistent pattern. In a further 8 patients studied by the same group [7], postoperative activations of sensorimotor areas normalized to become similar to healthy controls after CSM decompression surgery. In contrast, a study by Duggal et al. [29] in CSM patients demonstrated a larger volume of activation within the precentral motor areas and reduced volume of activation in the postcentral areas before operation. Postoperatively, continued enlarging volumes of activation were noted in both these areas regions of interest. In summary, while most fMRI evidence points to increased activation of motor areas in CSM before operation, the findings postoperatively did not indicate a uniform pattern of activation. The underlying reasons remain unclear, and further investigation, particularly in conjunction with electrophysiological or neurobiological methods, is justified.

Neurobiological evidence certainly exits with regard to the axonal sprouting and contacting of propriospinal neurons in animal experiments after transection of corticospinal projection to the hind limbs [30]. Additionally, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 delivered to corticospinal neurons resulted in increased collateral sprouting and contacts with propriospinal neurons [31, 32]. In somatosensory deprived rats by thoracic cord transections, upregulation of BDNF and decrease of gene activity for Nogo receptor were also demonstrated [33]. In another experiment, intrathecal Nogo receptor antagonist promoted growth of corticospinal axons in lesioned rats [34]. Collectively, these findings provide evidence to further support clinical, electrophysiological, and imaging data, suggesting modulation of neuroplasticity in response to spinal cord lesions.

In summary, TMS mapping of the motor cortex is well recognized to reflect functional plasticity of cortical outputs topographically [35]. The technique has been utilized to investigate cortical reorganization with training tasks, stroke rehabilitation, and peripheral limb amputation. While there is no universally standardized technique for motor mapping, MEP amplitudes and number of excitable sites [36–38] over a grid area [39] have been used extensively as mapping parameters. The methodology has been found to be robust and stable over time [40].

Before decompressive surgery, increased cortical representation of intrinsic hand muscle compared to normal

controls is not unexpected and likely reflects an inherent compensatory mechanism in response to cord compromise. The observation is corroborated by functional imaging in spinal cord injury [25, 26] and CSM [16] as well as animal models [30–33]. However, postoperatively, the tendency to normalization of motor representation is less well understood and inconsistent [29] but may be best explained in relation to recovery from chronic partial spinal cord injury. Like spinal cord injury, CSM can be heterogeneous, and it may be crucial but difficult to distinguish natural recovery compensatory mechanisms and that due to therapeutic intervention, such as motor training. Even in the present study whereby all patients do not receive physiotherapy, postoperative motor activity can be different for each patient, and standardization will be challenging over a 4-month period. Additionally, in the recovery period, the extent of synaptic transmission and reorganization is dependent on time after the initial insult to the spinal cord [41] as well as the variable degree of residual spinal cord atrophy [42]. While our findings point to reduction and normalization of motor representation 4 months after surgery, the findings cannot be reliably corroborated with published imaging studies in view of differences in follow-up duration and lack of a repeat MRI in most studies to ascertain cord atrophy.

In CSM, compression of descending corticospinal tracts results in desynchronization of I-wave volleys evoked with single pulse TMS of the primary motor cortex. The MEPs obtained can be used to calculate the CMCT by subtracting the peripheral conduction time. CMCT is more sensitive measure of corticospinal dysfunction in CSM than somatosensory evoked potentials [43–45] and can be utilized for the presurgical evaluation of CSM patients in the clinical setting [17]. In a prospective study of 141 CSM patients, excellent correlation of MRI with CMCT in terms of sensitivity and specificity was demonstrated [22]. Another prospective study of 241 patients found that TMS parameters had 98% sensitivity and specificity for mild cord compression, suggesting that TMS can be employed as a screening tool in CSM before MRI [2].

Noteworthy though, we did not find significant CMCT changes before and after surgery in all 4 limbs, despite motor cortex excitability modulation evident with cortical mapping as well as improvement in hand function in relation to MEP changes. In line with these observations, modulation of the ability to facilitate horizontal rather than vertical synaptic connections would be the most likely underlying mechanism at play. As TMS largely stimulates cortical neurons in a transsynaptic fashion [46], motor mapping with TMS will likely yield the most valid information in terms of plasticity changes. To our knowledge, this has only been studied in the context of SCI. Streletz et al., using serial motor mapping of C5 to C6 SCI patients, showed that enlarged contralateral biceps representation was present as early as Day 6 after injury [47]. In contrast, a separate study of 22 SCI patients using TMS did not show significant map changes after injury [48]. Similar to functional imaging, it can thus be appreciated that TMS motor mapping after cord dysfunction also did not yield findings with uniform characteristics. To our knowledge, studies of this nature have not been performed in CSM pre- or postoperatively. CMCT is the most frequently used and sensitive electrophysiological parameter to evaluate CSM clinically, and its methodology is fairly standardized across clinicians and researchers. CMCT evaluates motor cortex to anterior horn cell conduction and reflects integrity of rapid, direct descending pyramidal connections to the same intrinsic hand muscle (FDI) used for motor mapping in the present study. This further adds to the validity of our observations that lack of CMCT changes postoperatively implies modulation of the ability to facilitate horizontal rather than vertical synaptic connections as the most likely underlying mechanism at play.

The lack of H reflex modulation despite significant TMS mapping changes suggests that supraspinal rather than spinal mechanisms are predominant in driving plasticity after surgery. These findings are also in line with our previous impression that horizontally orientated cortical elements are largely responsible with observed TMS motor mapping changes. Modulation of the H reflex is well known to be reflective of changes in spinal excitability [49]. It has been used to assess spinal interneuronal excitability at rest and even during movement [50] as well as in combination with TMS efficaciously [51]. Although the H amplitudes and H/M ratios [51] are largely contributed by monosynaptic Ia excitation of spinal motor neurons [52], other mechanisms, including reciprocal and Ib inhibition, are known to modulate H reflex characteristics. Thus, it is imperative that recording conditions must be standardized to allow for a relaxed patient in quiet experimental conditions, delivering fixed stimulation parameters.

In the light of current knowledge outlined above, it is imperative that our findings can be applied to elucidate modulation of cortical motor control mechanisms in CSM. Based on comparison with healthy controls and within each patient, compensatory expansion of the hand area, in terms of magnitude and spatial representation of cortical excitability postoperatively, is evident. These observations are further strengthened by findings that, for Group A patients, both magnitude and spatial characteristics were larger than controls, whereas for Group B, only spatial characteristic were. This may be related to Group A patients having relatively more upper limb motor deficits compared with Group B, hence, driving enhanced cortical compensatory representation [53]. Furthermore, postoperatively, reduction in magnitude and spatial characteristics of cortical excitability were seen only in Group A, reflecting, for similar reasons, compensatory changes more specific to upper limb functional deficits, compared to Group B patients with lower limb dysfunction exclusively.

We next examined cortical excitability modulation in relation to the functional relevance of these changes. In terms of objective hand function tests, significantly increased grip strength and reduced lifting time for small objects rather than the other tests likely reflected improved direct projections for intrinsic hand musculature. However, significant correlation of changes in magnitude of cortical excitability for both feeding and stacking objects also likely reflects participation of more proximal muscles needed for these tasks which were modulated in terms of horizontal placed connectivity

postoperatively. Similarly, spatial changes in terms of number of excitable sites during TMS correlating with writing tasks also reflected functional cortical participation for both intrinsic muscle and wrist action, corroborating the experimental design and TMS both evaluating predominantly motor representation of distal muscles performing more finely skilled tasks.

All these observations, again, were seen exclusively in Group A patients, and all hand function tests were designed to evaluate the upper limb only. It would be interesting to compare our findings with the only fMRI study to date incorporating hand function tests [7]. In the 3-finger pinch tests, pinch-related activation volume in the ipsilateral sensorimotor cortex and the magnitude of activation in the contralateral dorsal premotor cortex evolved linearly across time after surgery, along with wrist extension-related activation magnitude in the contralateral supplementary motor area. However, in contrast to our findings which suggested reduction and return to normalcy of cortical excitability after surgery, there was no unidirectional change noted. The exact reasons are unclear, but the two studies employ different evaluation methods, as well as nonidentical hand function tasks which may partially explain differential results.

It is noteworthy that current knowledge may be limited by several factors. For fMRI, tasks are often limited to motor imagery rather than actual muscle activation due to the presence of movement artefacts. For electrophysiological studies, however, both resting and active tasks can be studied. In an event when both functional imaging and TMS results must be combined, it should thus be noted that findings may not be directly comparable. Overall, published studies are usually small in subject numbers, lacking in standardization of protocols and serialization of data. These deficiencies should be addressed in larger future studies of a similar nature.

In conclusion, we have demonstrated that compensatory expansion of motor cortical representation with a tendency to normalization after surgery occurs largely at cortical rather than spinal level. Cortical plasticity modulation mirrored improvements in relevant tasks requiring utilization of predominantly distal hand muscles. These findings have important implications with regard to the understanding and rehabilitation of patients with lesions involving the cervical spinal cord.

Conflict of Interests

There is no conflict of interests or financial disclosure for all authors.

Authors' Contribution

Andrew Green contributed in manuscript concept, data acquisition, and manuscript preparation. Priscilia W. T. Cheong contributed in data acquisition. Stephanie Fook-Chong helped in data analysis. Rajendra Tiruchelvarayan helped in data acquisition. Chang Ming Guo helped in data acquisition. Wai Mun Yue helped in data acquisition. John Chen helped in data acquisition. Yew Long Lo contributed in manuscript concept, data acquisition, and manuscript preparation.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 503079, 10 pages
http://dx.doi.org/10.1155/2015/503079

Research Article

Alterations of the Ceramide Metabolism in the Peri-Infarct Cortex Are Independent of the Sphingomyelinase Pathway and Not Influenced by the Acid Sphingomyelinase Inhibitor Fluoxetine

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Received 30 January 2015; Accepted 6 April 2015

Academic Editor: Michael S. Beattie

Ceramides induce important intracellular signaling pathways, modulating proliferation, migration, apoptosis, and inflammation. However, the relevance of the ceramide metabolism in the reconvalescence phase after stroke is unclear. Besides its well-known property as a selective serotonin reuptake inhibitor, fluoxetine has been reported to inhibit the acid sphingomyelinase (ASM), a key regulator of ceramide levels which derives ceramide from sphingomyelin. Furthermore, fluoxetine has shown therapeutic potential in a randomized controlled rehabilitation trial in stroke patients. Our aim was to investigate and modulate ceramide concentrations in the peri-infarct cortex, whose morphological and functional properties correlate with long-term functional outcome in stroke. We show that certain ceramide species are modulated after experimental stroke and that these changes do not result from alterations of ASM activity, but rather from nontranscriptional induction of the ceramide *de novo* pathway. Unexpectedly, although reducing lesion size, fluoxetine did not improve functional outcome in our model and had no significant influence on ASM activity or the concentration of ceramides. The ceramide metabolism could emerge as a potential therapeutic target in the reconvalescence phase after stroke, as its accumulation in the peri-infarct cortex potentially influences membrane functions as well as signaling events in the tissue essential for neurological recovery.

1. Introduction

Stroke is a disease of enormous socioeconomic relevance. Worldwide, it is the second leading cause of death and the leading cause of adult disability [1]. So far, acute stroke therapies aim at recanalizing the occluded brain vessels by means of pharmacologic thrombolysis or thrombectomy. Decades of research on (neuro)protective drugs have established several promising candidates that reduced infarct size in experimental animal models of stroke with positive effects on neurological outcome in short term observations. However, none of these substances could prove efficiency in large scale randomized controlled trials in stroke patients. A relatively new experimental approach is to target the dysfunction and mechanism of recovery within the nonischemic tissue surrounding the infarcted area, the so-called peri-infarct cortex. The peri-infarct tissue of the photothrombotic stroke model shows high morphological similarities to the penumbra in other stroke models such as distal middle cerebral artery occlusion, but most importantly, this model allows the investigation of long-term functional outcome in mice [2].

Sphingolipids are a complex class of signaling molecules and an essential part of cellular membranes. Their cellular levels regulate proliferation, apoptosis, and inflammation depending on the specific sphingolipid species, cell and receptor type, and different intracellular targets [3]. Ceramides are the backbone of more complex sphingolipids and the precursor of the versatile signaling molecule sphingosine-1-phosphate. They are essential for specific membrane functions (e.g., the formation of lipid rafts and caveolae) [3] and directly modulate intracellular effector proteins such as PKCζ [4], c-Raf [5], and CAPP [6]. Ceramide is tightly regulated in the cells, and its participation in cell death signaling pathways is controlled by rapid conversion of ceramide into less noxious/toxic sphingolipids. Ceramides are generated in different cellular compartments by three different pathways (Supplemental Figure 3 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/503079): the *de novo* pathway in the endoplasmatic reticulum, the salvage/sphingomyelinase pathway in the Golgi, lysosome, and the plasma membrane, as well as by recycling of glycosphingolipids. The cellular function of ceramides is in part dependent on the chain length, which is determined by differential activity of specific ceramide synthases (CerS 1–6) [7].

There is some evidence that alterations in sphingolipid metabolism, leading to enhanced ceramide production, occur in neurological disorders, such as multiple sclerosis [8], Wilson's disease [9], and Alzheimer's disease [10]. Cytokines such as tumor necrosis factor-alpha (TNF-α) [11] and reactive oxygen species (ROS) [12] induce the production of ceramide through activation of sphingomyelinases in this context. Therefore, the activation of the sphingomyelinase pathway is believed to be a general cellular stress response.

In experimental stroke, there are several studies showing an increase in ceramide synthesis via higher acid sphingomyelinase (ASM) activity [13–15] directly within the ischemic lesion. However, these studies did not investigate the relevance for long-term functional outcome as they focused on the ischemic, death prone tissue and a short observation period. In that context, an experimental reduction of ceramide production was shown to be (neuro)protective leading to smaller infarct sizes and better short term outcome.

Intriguingly, the antidepressant fluoxetine, a selective serotonin reuptake inhibitor and the only drug which has been shown to improve poststroke motor recovery in a randomized controlled neurorehabilitation trial in stroke patients [16], has recently been shown to inhibit ASM activity in the rodent healthy brain [17]. As the different functions of ceramide indicate a possible role in peri-infarct inflammatory, degenerative, and recovery processes, we hypothesized that ceramides are relevant for long-term functional outcome after stroke. Given the potential of fluoxetine to inhibit the ASM, the prorehabilitative effect of fluoxetine in stroke recovery might be attributable to the modulation of ceramide levels in the peri-infarct cortex.

We therefore hypothesized that (a) the peri-infarct region shows alterations of ceramide levels, that (b) these alterations result from a change of ASM activity, and (c) that fluoxetine improves motor recovery after stroke, and this effect might be mediated by ASM inhibition.

2. Methods

2.1. Photothrombotic Stroke and Drug Administration and Behavioral Testing. All animal experiments were approved by the local government authorities (Regierungspraesidium Darmstadt). Sample size calculations for all experiments were performed, assuming a power of 80%, previously published standard deviations and a difference between treatment groups of 20%. Photothrombosis (PT) was performed as previously described [18]. Briefly, 6–8 week old C57/Bl6 mice were anaesthetized by 1.7% isoflurane and 0.1 mg/kg buprenorphine s.c. and placed into a stereotactical frame. Five minutes after injection of 0.2 mL rose-bengal i.p. (Sigma-Aldrich, Taufkirchen, Germany; 10 mg/mL), the skull was illuminated at the motor cortex by a cold light source. At days 1, 3, 7, and 28, respectively, 10 mice per group (sham, saline, and fluoxetine) were killed by a lethal dose of isoflurane and immediate transcardial perfusion with 0.9% saline. After measurement of stroke size by either TTC staining or a photograph of the brain surface, 10 mg blocks of the peri-infarct cortex (Supplemental Figure 1) were dissected and immediately frozen in liquid nitrogen. At days −7, 7, and 28, behavioral outcome was determined by the cylinder task and the grid-walking test as previously described [19]. The observer was blinded for treatment groups. Fluoxetine (ratiopharm, Ulm, Germany) was given from day 3 to day 28 after stroke via drinking water (concentration: 120 mg/L) as previously published [17]. Fluoxetine plasma-levels were measured at day 7 and 28 (Supplemental Method 1).

2.2. LC-MS/MS. For quantification of ceramides, their precursors and metabolites, about 10 mg tissue was homogenized with PBS and liquid-liquid extracted with methanol : chloroform : HCl (15 : 83 : 2). The analytical procedure was similar to the method published elsewhere ([20] see Supplemental Method 2).

2.3. Sphingomyelinase Activity. The samples were lysed in 250 mM sodium acetate (pH 5.0), 1% NP40 and 1.3 mM EDTA. The tissue was then homogenized using the TissueLyser LT (1 min, 50 Hz; Qiagen, Hilden, Germany). Aliquots of the lysates were diluted to 250 mM sodium acetate (pH 5.0), 0.1% NP40, and 1.3 mM EDTA, then incubated with 10 nCi per sample [^{14}C]sphingomyelin for 10 min at 37°C. The reaction was stopped by addition of 600 μL of chloroform/methanol (2 : 1), and phases were separated by centrifugation. Radioactivity of the aqueous phase was quantified by scintillation counting and enabled quantification of ASM activity. For the NSM assay another lysis-buffer (HEPES 100 mM; 0.1% NP40; 5 mM DTT; 10 mM $MgCL_2$; 1.4 mM EDTA), reaction, and suspension buffer (HEPES 100 mM; 0.1% NP40; 5 mM DTT; 10 mM $MgCL_2$) were used. Substrate concentration, protein concentration, and reaction time were chosen after pilot studies (Supplemental Figures 2C and D).

2.4. RT-PCR. After homogenization using the TissueLyser LT (1 min, 50 Hz; Qiagen, Hilden, Germany), 1.2 μg of total RNA was isolated with TRIZOL (Sigma-Aldrich, Steinheim, Germany) according to the manufacture's protocol and used for reverse transcriptase-polymerase chain reaction (RT-PCR; Revert Aid first strand cDNA synthesis kit, Thermo Fisher Scientific, St. Leon-Rot. Germany) utilizing an oligo (dT) primer for amplification.

Real-time PCR (TaqMan) was performed using Applied Biosystems 7500 Fast Real-Time PCR System. Probes, primers, and the reporter dyes 6-FAM and VIC were from Life Technologies (Darmstadt, Germany). The cycling conditions were as follows: 95°C for 15 min (1 cycle), 95°C for 15 s, and 60°C for 1 min (40 cycles). The threshold cycle (C_t) was calculated by the instrument software (7500 Fast System SDS Software version 1.4). Analysis of the relative mRNA expression was performed using the $\Delta\Delta C_t$ method. The housekeeping gene GAPDH was used for normalization.

3. Results

Mass spectrometry revealed a reduction of total ceramide levels in the peri-infarct cortex (Figure 1(a)) at day 1 after photothrombosis (77.19% ± 15.51) compared to the corresponding cortex area in sham-operated mice (100% ± 21.5, $p = 0.02$, $n = 8$–10). However, at day 3 we found a significant increase of total ceramide (170% ± 39.79 versus 100% ± 24.85, $p = 0.0003$, $n = 8$–10), which persisted up to day 7 (140% ± 29.58 versus 100% ± 23.8, $p = 0.0035$, $n = 10$). Interestingly, the direct ceramide precursor of the *de novo* pathway, dihydroceramide (DHC, Figure 1(b), Supplemental Figure 3), was correspondingly increased at day 3 (258.5% ± 85 versus 100% ± 14.74, $p < 0.0001$, $n = 8$–10) and day 7 (180% ± 52 versus 100% ± 12.4, $p = 0.0002$, $n = 10$). Both ceramide and DHC normalized compared to sham at day 28. The precursor of DHC is sphinganine (Figure 1(c)), which was increased at day 7 after photothrombosis (147% ± 58.62 versus 100% ± 19.12, $p = 0.0274$, $n = 10$). Sphingosine (Figure 1(d)), the precursor as well as derivate of ceramide via the CerS or the ceramidases, but present at much lower concentrations than ceramide [3], was found to be decreased at day 1 (78% ± 16.46 versus 100% ± 16.26, $p = 0.0115$, $n = 8$–10) but increased at day 3 (119% ± 17.47 versus 100% ± 9.4, $n = 8$–10, $p = 0.0083$) and day 7 (191% ± 95.89 versus 100% ± 12.3, $p = 0.0083$, $n = 10$). Next we checked for an effect of stroke on the glycosphingolipid metabolites. Total glucosylceramides (Figure 1(e)), which can be both a precursor of ceramide via the glucocerebrosidase (GBA) and a product of the glucosylceramide synthase, was reduced at day 3 (63% ± 28.21 versus 100% ± 29.09, $n = 8$–10, $p = 0.0146$). Total lactosylceramides however (Figure 1(f)) were elevated at day 7 (232% ± 92.48 versus 100% ± 36.05, $n = 10$, $p = 0.0005$).

In the peri-infarct cortex, ceramide subspecies levels were differentially changed (Figure 2). Ceramide 18:0 is by far the most abundant ceramide species in the CNS [21] and contributed as the major part of the here observed ceramide elevation. Therefore, its dynamic reproduces that of total ceramides. It was reduced at day 1 compared to sham (Figure 2(a), 76% ± 18.06 versus 100% ± 23.59, $p = 0.0285$, $n = 8$–10) and then increased at day 3 (181% ± 46.97 versus 100% ± 24.68, $p = 0.0009$, $n = 8$–10). Ceramide 16:0 was decreased at day 1 (72% ± 15.33 versus 100% ± 23.52, $p = 0.0096$) then increased at day 3 (290% ± 106 versus 100% ± 29.02, $p < 0.0001$, $n = 8$–10) and day 7, but normalized at day 28. Ceramide 18:1 and ceramide 20:0 behaved in similar fashion (data not shown). The very-long chain ceramides 24:0 and 24:1 however increased only at a later time point at day 7 (238.7% ± 136.1 versus 100% ± 15.71, $p = 0.0053$, $n = 8$–10, ceramide 24:0). All measured ceramide subspecies concentrations returned to baseline at day 28. This resulted in changes of the ratios of ceramides with long chain length to ceramides with very-long chain length (ceramide 16:0/ceramide 24:0 + ceramide 24:1, Figure 2(b)) compared between day 3 and day 7 (0.69 ± 0.23 versus 0.47 ± 0.74, ANOVA, mean difference 0.2095, 95% CI of diff. 0.01397 to 0.4051, $n = 8$–10).

As our hypothesis was relying on the assumption that the sphingomyelinase pathway is upregulated under proinflammatory and hypoxic conditions, we measured sphingomyelinase activity in the peri-infarct cortex. However, the activity of both ASM and the NSM were unchanged (Figure 3); ASM activity was higher compared to NSM activity.

In order to investigate a potential transcriptional regulation of the different ceramide generation pathways, we performed Taqman PCR of the most relevant enzymes (Supplemental Figure 3). Against our hypothesis but consistent with enzyme activity, we found a reduction of ASM mRNA (Figure 4(a)) at day 7 (85% ± 13.21 versus 100% ± 12.73, $n = 8$–10, $p = 0.0312$), and of the neutral sphingomyelinase 2 (NSM-2, Figure 4(b)) at day 3 (62% ± 20.12 versus 100% ± 21.88, $n = 9$-10, $p = 0.0012$) and day 7 (66% ± 16.77 versus 100% ± 24.82, $n = 9$-10, $p = 0.0026$). NSM-1 mRNA (Figure 4(c)), which is present in much lower quantities than the NSM-2 mRNA (data not shown) was unchanged, as well as the acid ceramidase (ACER) mRNA (Figure 4(d)), the neutral ceramidase (NCER) mRNA (Figure 4(e)), and the acid glucocerebrosidase 1 (GBA1) mRNA (Figure 4(f)). GBA2 mRNA (Figure 4(g)) was reduced at day 1 (76% ± 25 versus 100% ± 17.59, $n = 9$, $p = 0.0297$). CerS1 (Figure 4(h)) is mainly responsible for the *de novo* synthesis of ceramide 18:0 and 20:0 and is downregulated at day 7 (66% ± 20.73 versus 100% ± 25.65, $n = 9$, $p = 0.026$). CerS2 (Figure 4(i)) produces ceramide 20:0 to 26:0 and is downregulated at day 1 (74% ± 23.65 versus 100% ± 24.41, $n = 9$, $p = 0.0335$). CerS4 (responsible for ceramide 18:0 and 20:0) and CerS6 (ceramide 14:0, 16:0 and 18:0) were unchanged (Figures 4(j) and 4(l)), whereas CerS5 (ceramide 16:0) was upregulated at day 1 (132% ± 30.12 versus 100% ± 15.71, $n = 9$, $p = 0.0120$).

Nevertheless we anticipated a reduction of ceramide levels by inhibiting the acid sphingomyelinase pathway by treatment with the functional inhibitor fluoxetine, starting at day 3 after photothrombosis. We found an effect of fluoxetine on stroke/glial scar size at day 28 (1.047 mm^2± 0.33 versus 2.778 mm^2± 0.55, $p < 0.0001$, $n = 10$; Figure 5(a)) but could not observe any effect of fluoxetine on total ceramide levels (Figure 5(d)) and ASM activity (Figure 5(e))

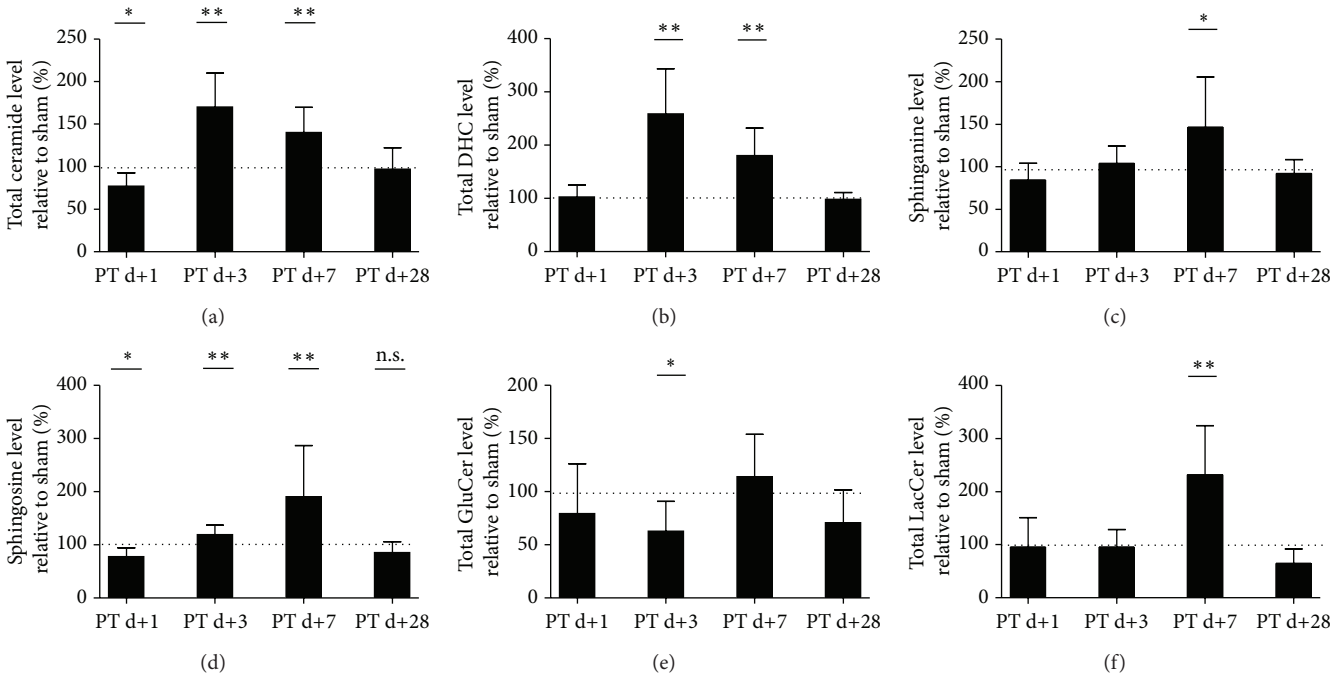

FIGURE 1: Ceramide, its precursors, and its derivate levels are altered in the peri-infarct cortex after photothrombotic stroke. (a) Total ceramide levels, (b) dihydroceramide levels, (c) sphinganine levels, (d) sphingosine levels, (e) glucosylceramide levels, and (f) lactosylceramide levels. Sphingolipids were measured at the indicated time points by tandem mass spectrometry. Differences between sham and PT group were analyzed using Student's unpaired two-tailed t-test. Data are presented as means ± SD; sham values are indicated by dotted line (for individual sham-SD's, see Section 3); values are not significantly different compared to sham if not marked otherwise; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; n.s., nonsignificant; $n = 8$–10/group.

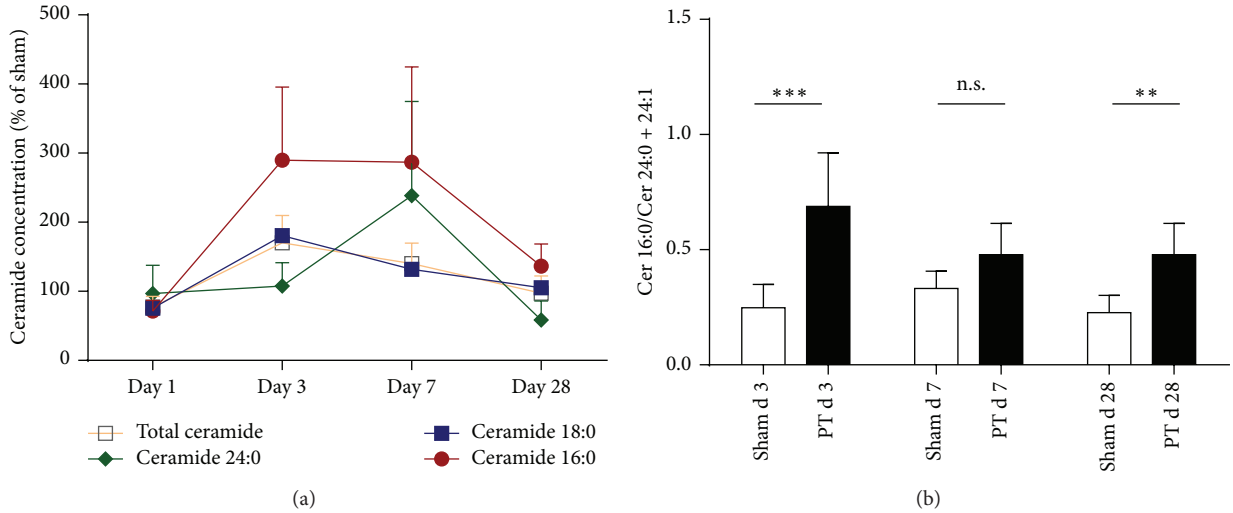

FIGURE 2: Ceramide subspecies are differentially regulated in the peri-infarct cortex. (a) Total ceramide, ceramide 16:0, 18:0, and 24:0 in the time course after photothrombotic stroke compared to sham. Asterisks for p values as well as ceramide 18:1 and 24:1 are not shown (see Section 3). (b) Ratios for ceramide 16:0/ceramide 24:0 + 24:1. Data are presented as means ± SD. Differences between sham and PT group were analyzed using Student's unpaired two-tailed t-test. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$; n.s., nonsignificant; $n = 8$–10.

and most importantly on functional outcome (Figure 5(b)). As controls for the validity of our assay as well as the potency of fluoxetine and other antidepressants to inhibit the ASM at the concentrations we achieved in our mice, we measured ASM activity in vitro (Supplemental Figure 2A) and cortex of ASM homozygous and heterozygous knockout mice (Supplemental Figure 2B); in all cases, a reduction of ASM activity could be observed.

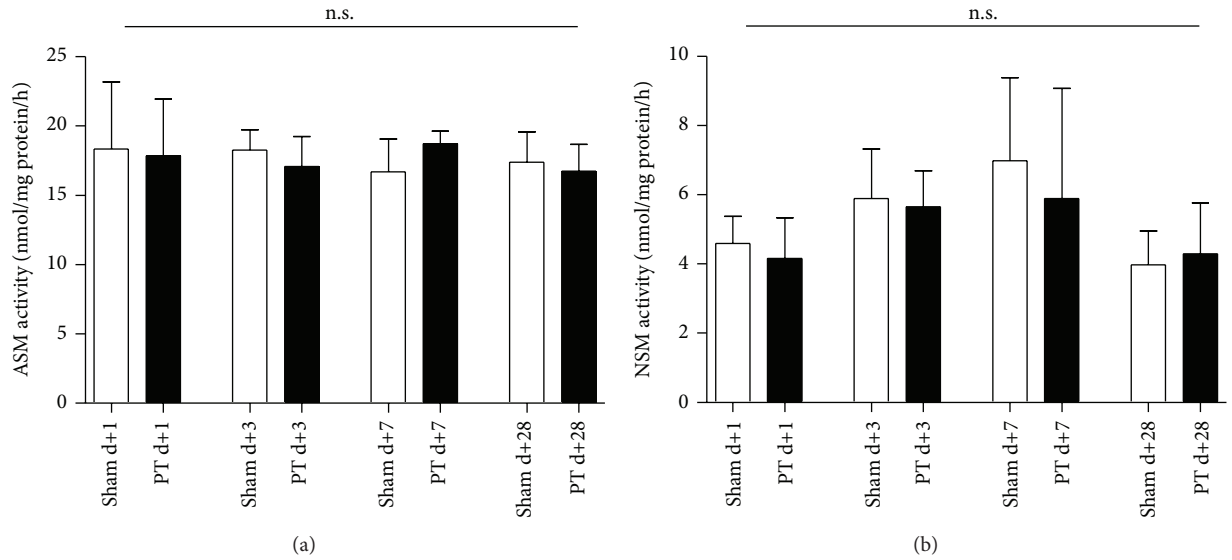

Figure 3: Sphingomyelinase activity is not altered in the peri-infarct cortex. (a) ASM activity and (b) NSM activity. Sphingomyelinase activity was measured at different time points by an enzyme activity assay with radioactive labeled sphingomyelin. Differences between sham and PT group were analyzed using Student's unpaired two-tailed t-test. Data are presented as means ± SD; n.s., nonsignificant; n = 5–10/group.

4. Discussion

The present study evaluated whether targeting ceramide-metabolism might be of potential therapeutic value in the poststroke reconvalescence period. We show a reduction of the bioactive sphingolipid ceramide in the subacute phase (day 1, Figure 1(a)) and an increase of ceramide in an intermediate time-window (day 3–7) within the peri-infarct region. Importantly, in this model, fluoxetine is not able to reduce sphingomyelinase activity, ceramide concentrations or to improve functional outcome. In order to determine other potential therapeutic targets, we investigated the ceramide synthesis pathways in the peri-infarct cortex and could show that the sphingomyelinase is not the responsible enzyme for the ceramide elevations but possibly changes in ceramide *de novo* synthesis.

This study has some major differences to previous studies, describing ceramides in experimental stroke, as these studies only investigated ceramide-levels in the infarct zone [13–15]. In ischemic tissue, the proapoptotic mechanism of increased sphingomyelinase activity has not only been shown in CNS, but also in various other cell types [22]. However, in the here investigated photothrombotic peri-infarct zone, apoptosis only takes place in a fine boundary zone and a short time window [23]. It is therefore unlikely that the elevation of ceramides we observed here, simply reflects mechanisms of cell death, especially as we surprisingly observed a decrease of total ceramide levels at 24 h post stroke, when relatively late penumbral apoptosis is supposed to be present and show only an increase from day 3 to day 7 after stroke. Diffusion from the ischemic core is rather unlikely due to ceramide's poor solubility. Importantly, we did not find any relevant changes of the acid and neutral sphingomyelinase activity (Figure 4) in the peri-infarct tissue compared to sham operated mice. Again, this is not comparable to previous data in experimental stroke, in which the induction of ASM-activity was a very early and short-term phenomenon [13–15]. In summary, we did not detect the very early increase of ASM-activity seen by others probably due to the analysis of different tissue and other time points.

Interestingly, although we found effects of late-onset fluoxetine treatment on infarct size and/or scar formation (Figure 5(a)), which is consistent with previous publications [24, 25], we did not see any effects of fluoxetine on sphingomyelinase activity, ceramide, or behavioral outcome (Figures 5(b)–5(e)). As this finding is a discrepancy to the previously reported effect of fluoxetine on ASM activity in C57/Bl6 mice [17], we performed various controls and could show the inhibition of ASM by fluoxetine in cell cultures, as well as a reduced ASM-activity in ASM homozygous knock-out animals (Supplemental Figure 2), validating the specificity and sensitivity of our assay. Furthermore, the plasma levels of the very lipophilic and blood-brain barrier penetrable drug fluoxetine were fairly high (day 7: 2.25 μM; day 28: 3.76 μM, n = 9-10). Unfortunately, we are not able to supply any well-known positive control of fluoxetine action in vivo, such as serotonin reuptake inhibition. However, the effect of fluoxetine on infarct size indicates that fluoxetine mediated some protective effect within the CNS. Additionally, our result is line with others, who could not show an effect of long-term treatment of mice with fluoxetine on ceramides in the CNS [26] and with previous data showing no effect of fluoxetine on recovery after stroke in rats [27, 28].

Our analysis included the direct precursors and derivate of ceramide (Supplemental Figure 3), allowing conclusions to be drawn to the involvement of other ceramide synthesis pathways. Exceptions are ceramide-1-phosphate, which

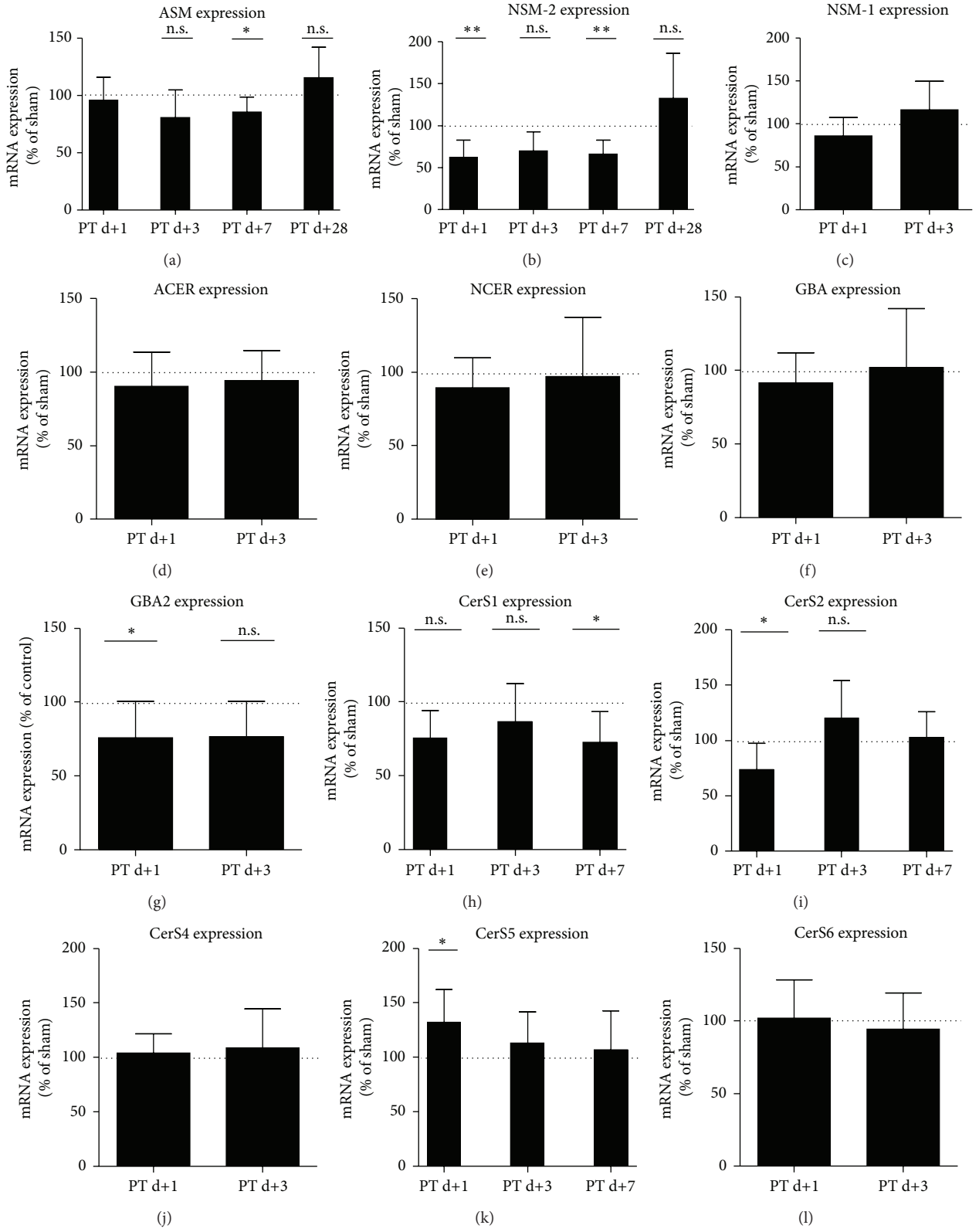

Figure 4: Expression of ceramide-metabolizing enzymes is differentially regulated in the peri-infarct cortex. (a) ASM mRNA, (b) neutral sphingomyelinase- (NSM-) 2 mRNA, (c) NSM-1 mRNA, (d) Acid ceramidase (ACER) mRNA, (e) neutral ceramidase (NCER) mRNA, (f) glucocerebrosidase- (GBA-) 1 mRNA, (g) GBA-2 mRNA, and (h)–(l) CerS1–6 mRNA, mRNA-levels were measured at the indicated time points by Taqman-PCR. Differences between sham and PT group were analyzed using Student's unpaired two-tailed t-test. Data are presented as means ± SD; sham values are indicated by dotted line (for individual sham-SD's, see Section 3); values are not significantly different compared to sham if not marked otherwise; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; n.s., nonsignificant; $n = 8$–10/group.

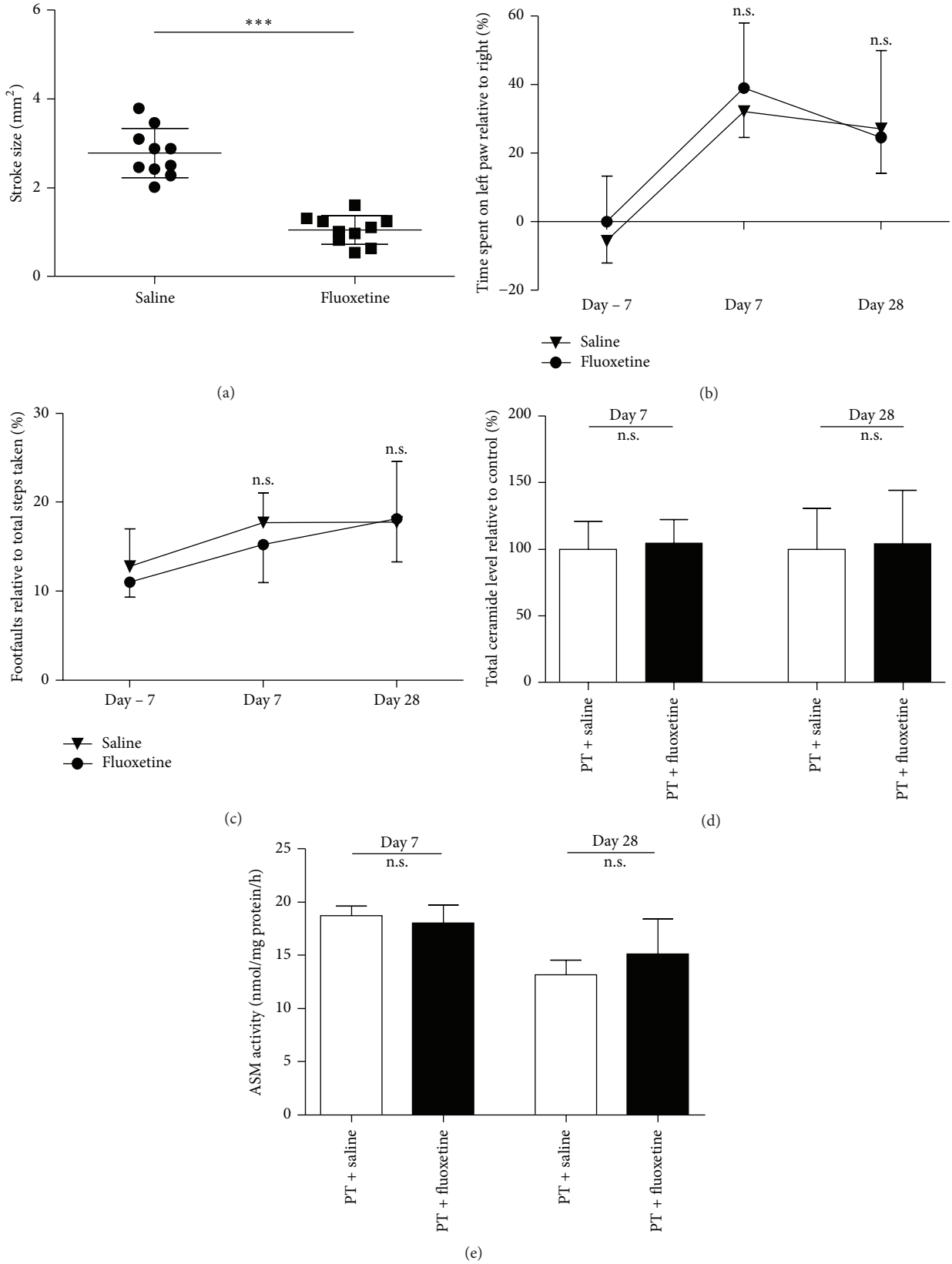

Figure 5: Fluoxetine treatment from day 3 to day 28 reduces infarct size, but has no impact on functional outcome, ASM activity or ceramide levels in the peri-infarct cortex. (a) Stroke size at day 28, (b) functional outcome, measured by the cylinder test, (c) functional outcome, measured by the forelimb grid-walking test, (d) total ceramide levels, and (e) ASM activity. Stroke size was determined by measuring the infarcted cortical area, ceramide level, ASM activity was measured at different time points as indicated above. Differences between saline and fluoxetine were analyzed using Student's unpaired two-tailed t-test. Data are presented as means ± SD; $^{***}p \leq 0.001$; n.s., nonsignificant; (a), (d), and (e): $n = 5$–10/group; (b) and (c): $n = 10$/group.

is present at a much lower quantity than ceramide and sphingomyelin, whose potential turnover was quantified by sphingomyelinase activity assays. The here observed increase of dihydroceramides (DHC) (Figure 1(b)) might indicate that instead of the sphingomyelinase pathway, the ceramide *de novo* pathway is upregulated within the peri-infarct tissue. Within this pathway, dihydroceramide (DHC) is the direct precursor of ceramide but also has biological signaling functions on its own [29] and importantly is not a derivate of ceramide. The serine-palmitoyl-Coa transferase (SPT) and the dihydroceramide desaturase (DES), both enzymes of the *de novo* pathway, are regulated by oxidative stress, hypoxia, and inflammation [30, 31]. However, the SPT is less likely to be the only responsible enzyme in the peri-infarct tissue, as we observed only an effect of stroke on sphinganine concentrations on day 7 (Figure 1(c)). As we show here, a parallel increase of ceramide and DHCs, the upregulation of ceramide synthesis probably involves enzymes more proximal than the DES. The enzymes distal of the SPT and proximal of the DES in the *de novo* pathway and responsible for synthesis of ceramide subspecies with specific chain lengths are the ceramide synthases [32]. Considering that our results show that an involvement of the SPT and the DES is less likely and that the increase of certain ceramide subspecies is responsible for the elevation of total ceramide, it is tempting to speculate that the ceramide synthases are responsible for the here observed elevation of ceramides. On the transcriptional level, most of the enzymes of the different ceramide synthesis pathways appear not to contribute to increased ceramide levels (Figure 4); only the increase of CerS5 mRNA (Figure 4(k)) at day 1 could explain the upregulation of Ceramide 16:0 ceramide at day 3–7. Furthermore, the decrease of CerS2 (Figure 4(i)), the second most common CerS in the CNS [33], could contribute to the decrease of total ceramide at day 1. However, it is important to consider, that discrepancies of ceramide synthase activity and transcriptional regulation has been shown previously [33, 34].

In the peri-infarct cortex, instead of cell death, events such as axonal sprouting, microglial and astrocytic activation, angio-/neurogenesis, and synaptic plasticity determine neurological outcome [2]. What could be the pathophysiological role of the observed increase of total ceramide and ceramide subspecies in the peri-infarct cortex? One has to keep in mind that most of the previous data about the effect of ceramide on CNS cells was determined in cell culture using short chain ceramide (Ceramide 2:0–6:0), which has very different properties compared to the more abundant long or very-long ceramides (Ceramide 16:0–24:0; [7]).

Concerning neuronal plasticity, PKCζ can be recruited and activated by ceramide [4, 35] and PKCζ has been shown to be important for long-term potentiation (LTP) creation and maintenance [36]. Ceramide-controlled lipid rafts are essential for efficient synaptic transmission, supporting an influence of ceramide levels on synaptic plasticity; for example, it has been shown that increasing ceramide levels increase NMDA receptor-mediated synaptic transmission [37, 38]. The increase of NMDA-receptor transmission facilitates long-term-potentiation and memory consolidation but on the other hand has a negative impact in the long-term by increasing excitotoxicity. In the case of recovery from experimental stroke, increased glutamatergic transmission in the peri-infarct cortex might be of advantage [19]. On the other hand, there is a negative influence of ceramide on axonal outgrowth, both by ceramide itself [39] and its metabolites [40]. Importantly, a recent publication shows that lactosylceramide controls astrocytic activation in an autocrine fashion, which indirectly influences microglial activity [41]. As we observe an increase of lactosylceramide at day 7 (Figure 1(f)), lactosylceramide may also play a role in (micro)glial activation in the peri-infarct cortex. In multiple sclerosis plaques, total ceramide has been shown to be reduced, but certain subspecies were increased within reactive astrocytes, contributing to blood-brain barrier damage [8]. Ceramide is an inhibitor of both neuro- and angiogenesis [17, 42], two important mechanisms for recovery after stroke [2]. Additionally, a more general neurotoxic effect of ceramide 16:0 and ceramide 24:0 could be recently shown [43].

There is some evidence that specific alterations of different ceramide subspecies occur in CNS diseases such as Alzheimer's disease [44]. This indicates that ceramides substantially influence pathophysiological processes in a manner dependent on the chain length. Concerning membrane functions, ceramide 16:0 and ceramide 24:0 have a different ability to form cluster with cholesterol [45], which is an essential step towards lipid raft formation [46] and clustering as well as activation of various proteins at the cell membrane [47]. Membranes of Cers2-KO-mice (with a lack of ceramide 24:0) are more fluid [48], which indicates that our data could have implications not only for cell membrane function, but also for the functionality of membrane bearing organelles in cells of the peri-infarct cortex. In the peri-infarct cortex we observed a differential regulation of ceramides with specific chain lengths (Figure 2), indicating a specific pathophysiological role for each ceramide subspecies. It appears as if the ceramide metabolism compensates the initially increased ratio of long to very long chain length at day 3 by upregulation of ceramide 24:0 and 24:1 leading to a transient, almost normalized ratio at day 7 (Figure 2(b)).

In summary, our observation of alterations of ceramide, its subspecies and metabolites in the peri-infarct cortex could have both a positive and a negative impact on recovery and function of the peri-infarct tissue after stroke. Unfortunately, the relevance of our observations remains to be proven, as we were not able to reduce ceramide generation by inhibition of sphingomyelinase activity. Future studies should address the potential targets pointed out in this study, that is, the ceramide *de novo* pathway, by other genetic and pharmacological approaches.

5. Conclusion

Our observation of an elevation of ceramide points to a complex, but well-coordinated pathophysiological role of sphingolipid metabolism in the peri-infarct cortex. This could have implications for our understanding of recovery from stroke and other acute CNS diseases and indicates potential therapeutic targets.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

R. Brunkhorst was supported by a stipend of the Medical Faculty of Goethe University (Patenschaftsmodell), the German Research Foundation (SFB 1039), and a publication grant of Neurowind. J. Pfeilschifter was supported by the German Research Foundation (SFB 1039, FOG 784, and PF361/7-1). J. Pfeilschifter and W. Pfeilschifter were supported by the Hans Kröner Graduate School. R. Brunkhorst, J. Pfeilschifter, and W. Pfeilschifter were supported by the Fondation Leducq (SphingoNet). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

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Hindawi Publishing Corporation
Neural Plasticity
Volume 2015, Article ID 823185, 10 pages
http://dx.doi.org/10.1155/2015/823185

Research Article

The Plasticity of Brain Gray Matter and White Matter following Lower Limb Amputation

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Received 9 January 2015; Accepted 25 March 2015

Academic Editor: Michael S. Beattie

Accumulating evidence has indicated that amputation induces functional reorganization in the sensory and motor cortices. However, the extent of structural changes after lower limb amputation in patients without phantom pain remains uncertain. We studied 17 adult patients with right lower limb amputation and 18 healthy control subjects using T1-weighted magnetic resonance imaging and diffusion tensor imaging. Cortical thickness and fractional anisotropy (FA) of white matter (WM) were investigated. In amputees, a thinning trend was seen in the left premotor cortex (PMC). Smaller clusters were also noted in the visual-to-motor regions. In addition, the amputees also exhibited a decreased FA in the right superior corona radiata and WM regions underlying the right temporal lobe and left PMC. Fiber tractography from these WM regions showed microstructural changes in the commissural fibers connecting the bilateral premotor cortices, compatible with the hypothesis that amputation can lead to a change in interhemispheric interactions. Finally, the lower limb amputees also displayed significant FA reduction in the right inferior frontooccipital fasciculus, which is negatively correlated with the time since amputation. In conclusion, our findings indicate that the amputation of lower limb could induce changes in the cortical representation of the missing limb and the underlying WM connections.

1. Introduction

Human brain plasticity or neuroplasticity refers to the capacity of the nervous system to modify the organization of the brain structure and function in response to experience. It is an intrinsic property of the nervous system retained throughout a lifespan [1]. Previous studies suggested that both short-term [2, 3] and long-term training [4–6] can modulate brain structural changes involved with both the gray matter (GM) and white matter (WM). The candidate mechanisms for these changes are multifaceted and likely include gliogenesis, synaptogenesis, and vascularization in GM, as well as myelination and axonal sprouting in WM [7].

In addition to normal training or experience, a growing body of evidence has accumulated supporting injury-induced functional or structural plasticity at different levels in the adult central nervous system [8–10]. Previous studies suggest that, at least in primates, plasticity in the cortical representation can occur rapidly as a consequence of peripheral lesions or sensory deprivation [11, 12]. As a drastic limb injury, amputation in humans has been reported to lead to extensive reorganization, most prominently in the primary somatosensory and motor areas, which was suggested to correlate with phantom limb pain (PLP) [13–16]. Despite extensive neurobiological research, the underlying nature of such phenomena remains elusive. While some authors have argued that cortical reorganization following amputation is triggered by the loss of sensory input [16, 17], others have proposed that the mechanisms should be attributed to the persistent experience of pain [18]. These discrepancies in the literature raise the fundamental question of whether brain reorganization occurs in amputees without PLP. On the other

hand, it also should not be overlooked that the short- and long-term effects of amputation on the brain may be varied, as PLP is usually more common in the initial stage after amputation [19].

Amputees have been found to have structural differences in both GM and WM. One study using automated voxel-based morphometric analysis found that subjects with limb amputation exhibited a GM decrease in the thalamus, which was unrelated to PLP [20]. However, this investigation did not distinguish between upper and lower limb amputation. In addition, reduced GM volume in the primary motor [21] or sensory [18] cortices was also observed in patients with amputation or spinal cord injury. In contrast to voxel-based morphometry, the measurement of cortical thickness provides a more direct and meaningful index. Preißler and colleagues [22] found that cortical thickness in upper limb amputees was reduced in the motor cortex but increased in the temporal and parietal lobes. Although GM reorganization was initially the focus of many brain imaging studies, WM changes after limb amputation are increasingly being investigated using neuroimaging techniques, especially diffusion tensor imaging (DTI), which provides information about WM tracts and their organization based on water diffusion. Fractional anisotropy (FA) is the most often used DTI index of WM integrity, and reduced FA in amputees has been reported in the corpus callosum (CC) and corticospinal tract [23]. Although these studies have been carried out to determine the effects of missing limbs on brain reorganization, little is known about the associations between GM and WM changes after amputation.

The purpose of this study was to examine the long-term patterns of brain reorganization following limb amputation. To systematically characterize brain reorganization, we first used a combined tract-based spatial statistics (TBSS) and tractography analysis, which enables a precise characterization of both whole-brain WM and specific anatomical fiber tracts, to assess the microstructural changes in patients with unilateral amputation in the lower limb. We then performed surface-based morphometry across the whole brain GM and regions of interest (ROI) focusing on the sensorimotor cortices. Finally, the relationships between GM and WM changes in amputees were investigated.

2. Materials and Methods

2.1. Subjects. Seventeen adult patients (13 males and 4 females) with right lower limb amputation were recruited from the Prosthetic and Orthotic Clinics at the Department of Rehabilitation, Southwest Hospital in Chongqing. All the patients had been fitted with prostheses. Twelve were amputations following traumatic injury and five were due to tumors (2 being melanoma and 3 being osteosarcoma). Ten amputations occurred at the transtibial and seven at transfemoral levels. Exclusion criteria were the following: (1) age at amputation of less than 18 years or more than 60 years; (2) amputation at another part of the body; (3) presence of major systemic disease (e.g., diabetes mellitus, cardiovascular diseases, and inflammation), psychiatric or neurological illnesses; (4) duration between amputation and magnetic resonance imaging (MRI) scanning of less than 6 months; (5) presence of PLP or stump pain assessed by the five-category verbal rating scale [24].

Eighteen age- and sex-matched healthy controls without neurological or psychiatric diseases and with normal brain MRI were recruited from the local community. All the participants were dominantly right-handed as determined by the Edinburgh Handedness Inventory [25] and had a score of 27 or higher on the Chinese version of the Mini-Mental Status Examination (MMSE) [26]. The study was approved by the Medical Research Ethics Committee of Southwest Hospital, and written informed consent was obtained from all participants.

2.2. Imaging Data Acquisition. All of the participants were scanned using a 3.0 Tesla imager (Tim Trio, Siemens, Erlangen, Germany) with a 12-channel head coil. DTI data were acquired using a single-shot twice-refocused spin-echo diffusion echo planar imaging sequence (repetition time = 10,000 ms, echo time = 92 ms, 64 nonlinear diffusion directions with $b = 1000\ \text{s/mm}^2$, and an additional volume with $b = 0\ \text{s/mm}^2$, matrix = 128×124, field of view = 256×248, and 2 mm slice thickness without gap). From each participant 75 axial slices were acquired and the diffusion sequence was repeated twice to increase the signal-to-noise ratio. T1-weighted three-dimensional magnetization-prepared rapid gradient echo images were then collected using the following parameters: repetition time = 1,900 ms, echo time = 2.52 ms, inversion time = 900 ms, flip angle = 9°, matrix = 256×256, thickness = 1.0 mm, and 176 slices with voxel size = $1 \times 1 \times 1\ \text{mm}^3$.

2.3. DTI Data Analysis. The DTI data were preprocessed using the FMRIB Software Library (University of Oxford, UK). First, the diffusion data were corrected for eddy currents and head motion, and the two acquisitions were averaged. The averaged images were masked to remove skull and nonbrain tissue using the FSL Brain Extraction Tool [27]. Then, the diffusion parametric images were calculated using the diffusion tensor analysis toolkit [28].

Data were then prepared for statistical analysis using TBSS [27]. First, FA images for all subjects were nonlinearly aligned to a study-specific minimal-deformation target (MDT) brain and resampled to an isotropic 1 mm resolution. The MDT brain was selected as the brain image that minimizes the deformation from other brain images in the group through warping all FA images in the group to each other [29, 30]. Next, the mean FA image was created and thinned to create a mean FA skeleton that represents the centers of all fiber tracts. The FA threshold of 0.2 was chosen to restrict the skeleton to WM tracts. Each subject's aligned FA data were then projected onto this skeleton.

2.4. Probabilistic Diffusion Tractography (PDT). Clusters showing group differences in the TBSS analysis were used as seed masks for multifiber probabilistic tractography [31] in each subject's native space. The steps have been described in

Table 1: Demographic characteristics of the participants.

	Patients	HC	*P* value
Age (years)	37.5 ± 13.5 (18–60)	37.0 ± 12.7 (19–60)	0.91
Male : female	13 : 4	13 : 5	0.54
Education level (years)	9.5 ± 2.7 (6–15)	9.6 ± 3.3 (5–16)	0.94
Age at amputation (years)	32.9 ± 12.6 (18–59)	—	—
Time since amputation (months)	71.4 ± 102.4 (7–336)	—	—
MMSE score	28.0 ± 1.4 (27–30)	28.4 ± 1.2 (27–30)	0.37

The data were presented as mean ± SD (range). HC, healthy controls; MMSE, Mini-Mental Status Examination.

detail in our previous articles [32, 33]. For each participant, PDT was run from each voxel in the seed mask to the whole brain using default parameters. The warp fields of nonlinear registration and the inverse versions were used for the translation between the original space and the standard space. For the elimination of spurious connections, the individual tracts in standard space obtained by PDT were arbitrarily thresholded to include only voxels through which at least 25% (1,250) of samples had passed. Each subject's tracts were then binarized and summed to produce group probability maps for each pathway. The group probability maps were also thresholded at 25% (at least 9 of the 35 subjects) to generate the masks for each fiber pathway. The WM labels atlas [34] and tractography atlas [35] implemented in FSL were used for the structural identification. Individual mean FA values of each pathway were then extracted from the standardized whole-brain DTI images.

2.5. Cortical Thickness Analysis. All the structural T1 images were analyzed using FreeSurfer (version 5.3.0, https://surfer.nmr.mgh.harvard.edu/) to create anatomical surface models. The automated processing stream mainly included removal of nonbrain tissue [36], Talairach transformation, segmentation of gray/white matter tissue [37], intensity normalization, topological correction of the cortical surface [38], and surface deformation to optimally place the tissue borders [39]. The tissue boundaries were reviewed and manually edited for technical accuracy. Cortical thickness was calculated as the shortest distance between the GM and WM surfaces at each vertex across the cortical mantle. Moreover, the GM volume in each hemisphere and total intracranial volume (TIV) was also calculated from the FreeSurfer processing stream.

Finally, using the Brodmann Areas (BA) atlas in FreeSurfer (https://surfer.nmr.mgh.harvard.edu/fswiki/BrodmannAreaMaps), we measured the individual mean cortical thickness values in the sensorimotor regions, including the bilateral BA 1, 2, 3a, 3b, 4a, 4p, and 6. In order to avoid the overlap among these labels, they were all thresholded at 80% probability.

2.6. Statistical Analyses. Group differences in age, years of education, and neuropsychological scores were examined using independent samples *t*-tests. Sex data were analyzed with a chi-square test. Differences in FA between the amputees and controls were determined using the FSL "randomize" tool, which is specifically designed for permutation testing with nonparametric values. Age and sex were used as the covariates. Clusters were reported reaching a significance level of $P < 0.05$, corrected for multiple comparisons across image using the null distribution of the maximum cluster mass ($t > 3$) [32]. Cluster mass is the sum of all statistic values within the cluster and has been reported to be more sensitive than cluster size [40].

Whole-brain vertex-wise group comparisons for cortical thickness were performed on a standardized surface [41] and the data were smoothed using a full-width/half-maximum Gaussian kernel of 10 mm on the surface. Regional differences between amputees and controls were assessed using a vertex-by-vertex general linear model controlling for the potential confounding effects of age, sex, and TIV. The statistical analyses were performed with the SurfStat toolbox based on Random Field Theory (RFT) [42]. Clusters were first reported reaching a significant level of RFT-corrected $P < 0.05$, and then those reaching a looser significance level of uncorrected $P < 0.005$ were also indicated.

Analyses of covariance (ANCOVA) adjusting for age and sex were used to explore the group differences in the mean FA value for each of the fiber tracts generated by PDT and in the mean cortical thickness for each of the selected sensorimotor regions in both hemispheres. Finally, the relationships between the WM and GM changes were investigated using partial correlation analyses (adjusted for age and sex). A false discovery rate (FDR) corrected threshold of 0.05 was considered as significant for these analyses.

3. Results

3.1. Demographics and Clinical Measures. Demographic and relevant clinical information is listed in Table 1. There were no significant differences in sex ratio, age, education, and MMSE scores between the amputees and controls.

3.2. WM Differences Revealed by TBSS and PDT. Compared with controls, the amputees showed a decreased FA in the right superior corona radiata and WM regions underlying the right temporal lobe and left premotor cortex (PMC) (Figures 1(a), 1(c), and 1(e); Table 2). No FA increase was found in amputees relative to controls.

PDT from the above clusters revealed that the contributing WM tracts were the commissural fibers connecting the

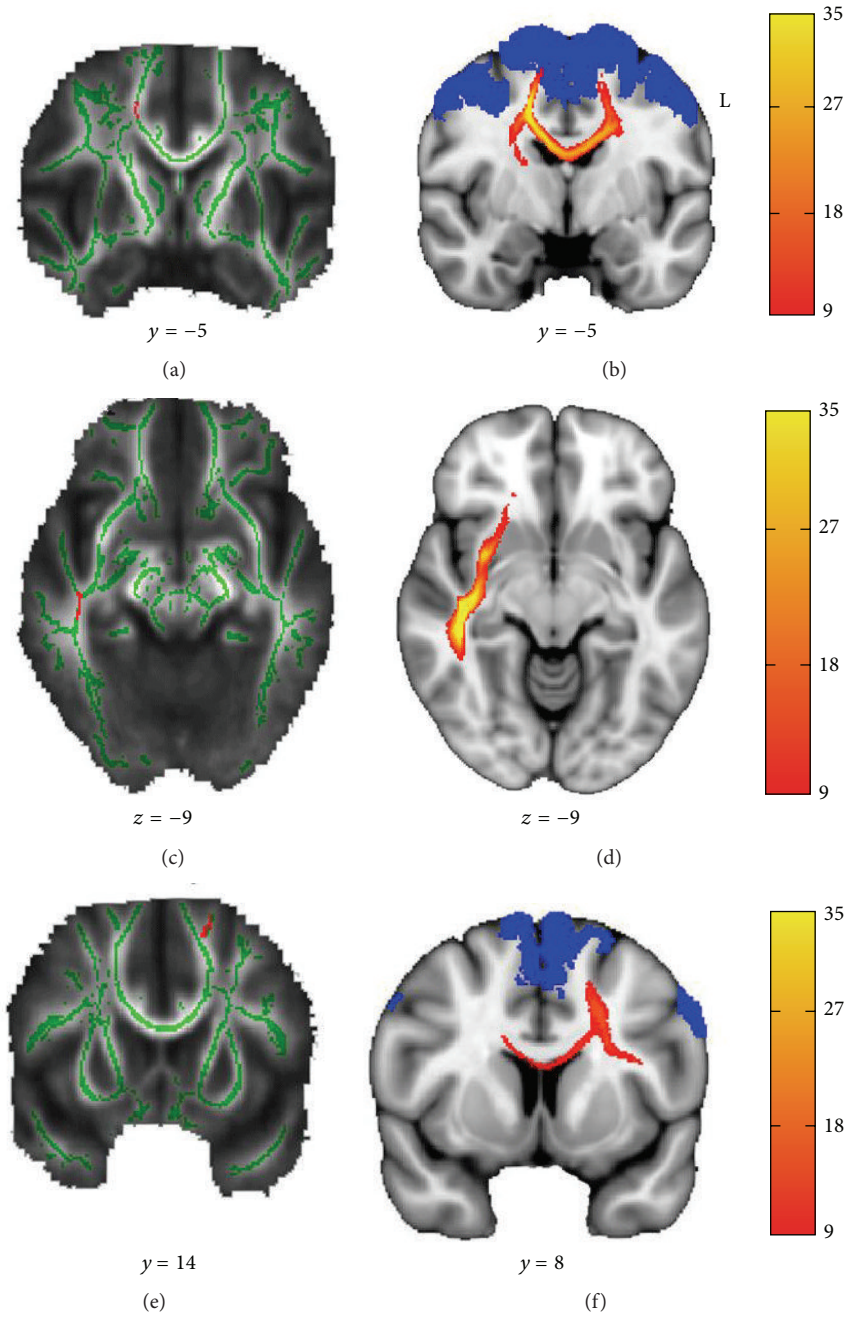

Figure 1: Results of TBSS analysis of FA maps (a, c, and e) and group probability maps (b, d, and f) from the corresponding regions. The mean white matter FA skeleton is shown in green. The blue mask indicates the PMC obtained from the Jülich histological atlas. The group probability maps were thresholded at 25% (at least 9 persons from the 35 subjects) and the color bar indicates the number of participants in whom the generating fiber pathways pass through that voxel.

Table 2: Regions showing significant FA reduction in the amputees.

Region	Cluster index	Hemisphere	MNI coordinates			Voxels	P value
			x	y	z		
Superior corona radiata	3	R	17	−6	38	105	0.03
Temporal WM	2	R	43	−24	−13	95	0.03
WM underlying PMC	1	L	−15	14	50	76	0.04

The output was thresholded at cluster level ($t > 3$) and corrected for multiple comparisons using the null distribution of the maximum (across image) cluster size ($P < 0.05$). MNI, Montreal Neurological Institute; PMC, premotor cortex; WM, white matter.

Table 3: The differences of FA values in fiber tracts generated from tractography.

Region	Cluster index	FA value		P value
		Controls	Patients	
WM connecting bilateral PMC	3	0.48 ± 0.02	0.44 ± 0.04	0.009
Right IFOF	2	0.47 ± 0.03	0.45 ± 0.02	0.009
WM underlying left PMC	1	0.36 ± 0.02	0.33 ± 0.02	0.0003

The P value was adjusted for multiple comparisons. IFOF, inferior frontooccipital fasciculus; PMC, premotor cortex; WM, white matter.

Table 4: Regions showing significant differences of cortical thickness across the whole brain.

Region	H	BA	Coordinates			Mean thickness		P value	Peak T score	Vertex number
			x	y	z	Patients	Controls			
PMC	L	6	−41	6	54	2.44 ± 0.40	2.81 ± 0.27	0.001	3.84	169
V1	R	17	12	−96	4	1.68 ± 0.16	1.89 ± 0.21	0.001	3.64	167
TOJ	R	37	43	−65	2	2.20 ± 0.15	2.45 ± 0.21	0.001	3.49	127
preCG	R	4	52	−8	43	2.62 ± 0.24	2.83 ± 0.16	0.001	3.55	108
V2/V3	R	18	30	−95	8	1.88 ± 0.21	2.14 ± 0.19	0.001	3.52	104
Precuneus	R	N.A.	16	−37	46	2.01 ± 0.17	2.22 ± 0.17	0.001	3.38	94
V1	L	17	−10	−88	6	1.42 ± 0.11	1.62 ± 0.19	0.003	3.40	89
IPL	L	7	−29	−71	40	2.12 ± 0.16	2.33 ± 0.16	0.001	3.76	86
OFC	L	47	−46	42	−10	1.94 ± 0.21	2.14 ± 0.21	0.002	3.17	59

The results were reported at $P < 0.005$ (uncorrected) and vertex number >50. BA, Brodmann Area; H, hemisphere; IPL, inferior parietal lobule; N.A., not available; OFC, orbital frontal cortex; PMC, premotor cortex; preCG, precentral gyrus; TOJ, temporooccipital junction; V1, primary visual cortex; V2/V3, extrastriate visual areas 2/3.

bilateral premotor cortices and the association fibers that exactly overlapped with the inferior frontooccipital fasciculus (IFOF) (Figures 1(b) and 1(d)). The cluster underlying the left PMC also generated local premotor and transcallosal paths (Figure 1(f)).

The results of ANCOVA demonstrated that the mean FA values extracted from the thresholded group probability maps in amputees were all significantly reduced ($P < 0.05$, FDR correction for multiple comparisons) in all the fiber tracts (Table 3).

3.3. Cortical Thickness Differences. The GM volume (controls versus amputees: left, 0.25 ± 0.02 versus 0.24 ± 0.03 L, $P = 0.13$; right, 0.25 ± 0.02 versus 0.24 ± 0.03 L, $P = 0.17$) and TIV (1.56 ± 0.15 versus 1.51 ± 0.14, $P = 0.17$) of amputees did not differ significantly from those of controls. The amputees showed a thinning trend ($P < 0.005$, uncorrected) in different cerebral lobules, with the largest one in the left PMC. Smaller clusters of cortical thinning were also noted in the bilateral occipital lobes, the right temporooccipital junction, precentral gyrus, precuneus lobe, the left inferior parietal lobule, and frontal orbital cortex (Figure 2; Table 4). However, no clusters survived after RFT correction for multiple comparisons. We did not find any clusters exhibiting thickness increase in the amputees compared with the control group ($P < 0.005$, uncorrected).

The results of ANCOVA for the ROI confirmed that the cortical thickness was only significantly decreased in the left premotor area (BA 6) in the amputees relative to the controls (2.73 ± 0.14 versus 2.84 ± 0.12; $P = 0.02$). The difference remained significant ($P = 0.03$) even when we added TIV as an extra covariate (Table 5).

3.4. Associations between WM and GM Changes in Amputees. No significant associations were found between the cortical thickness in the affected regions (as shown in Table 4) and the DTI parameters of the fiber tracts generated from the PDT in the amputees. However, partial correlation analyses revealed that the FA value of the IFOF (as shown in Figure 1(d)) was negatively correlated to the time since amputation ($r = -0.55$, $P = 0.03$).

4. Discussion

In the present study, we explored brain structural reorganization in lower limb amputees without PLP. Cortical thickness and FA values were used as measures to evaluate the GM and WM microstructural changes across the whole brain compared with normal controls. As a consequence, we found that patients with amputation at the right lower limb exhibited cortical thinning in the left premotor area and the right visual-to-motor regions. Additionally, the integrity of the fiber tracts connecting the bilateral PMC and those underlying the right visual-to-motor regions was also significantly reduced in the patients.

Our study demonstrates that cortical reorganization occurs in lower limb amputees, even in the absence of PLP. We observed a thinning trend in different cerebral lobules, especially in the PMC contralateral to the affected side. The PMC encompasses the anterior lip of the precentral gyrus,

Table 5: The differences of mean cortical thickness in the sensorimotor cortices between the amputees and normal controls.

Region	Hemisphere	Mean cortical thickness		P value[a]	P value[b]
		Controls	Patients		
BA 1	L	2.27 ± 0.22	2.16 ± 0.21	0.14	0.19
	R	2.25 ± 0.26	2.21 ± 0.21	0.65	0.73
BA 2	L	2.17 ± 0.19	2.08 ± 0.18	0.17	0.24
	R	2.11 ± 0.15	2.05 ± 0.17	0.27	0.46
BA 3a	L	1.67 ± 0.09	1.66 ± 0.10	0.79	0.86
	R	1.67 ± 0.10	1.65 ± 0.09	0.61	0.71
BA 3b	L	1.53 ± 0.09	1.49 ± 0.09	0.18	0.15
	R	1.54 ± 0.11	1.52 ± 0.12	0.6	0.61
BA 4a	L	2.74 ± 0.15	2.63 ± 0.21	0.06	0.09
	R	2.85 ± 0.19	2.77 ± 0.17	0.21	0.22
BA 4b	L	2.41 ± 0.17	2.31 ± 0.20	0.13	0.15
	R	2.39 ± 0.15	2.35 ± 0.20	0.53	0.40
BA 6	L	2.84 ± 0.12	2.73 ± 0.14	**0.02**	**0.03**
	R	2.83 ± 0.12	2.76 ± 0.18	0.20	0.23

[a]Adjusted for age and sex; [b]adjusted for age, sex, and total intracranial volume. BA, Brodmann Area; L, left; R, right. Bold indicates $P < 0.05$ (FDR correction for multiple comparisons).

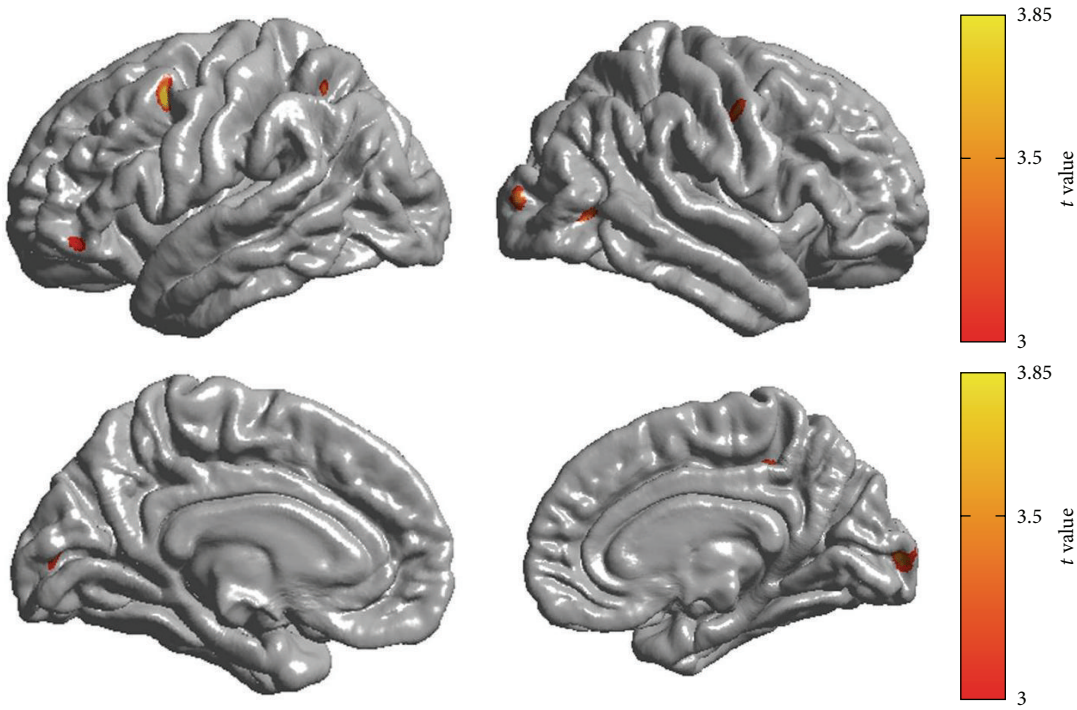

Figure 2: Regional cortical thinning in amputees compared with the controls. $P < 0.005$ ($t > 3$), uncorrected.

the posterior portion of the middle frontal gyrus, and the superior frontal gyrus on the superolateral surface of the brain, corresponding to part of BA 6 [43]. The time-specific studies of the PMC and primary motor cortex reflect the distinct roles of the two areas: the PMC is involved in movement selection, whereas the latter is involved in movement execution [44, 45]. The activity of PMC neurons is also responsible for the specification of movement parameters such as amplitude, direction, and speed of movement [43]. Additionally, the PMC also seems to be involved in the control of eye movements and eye-related neural activity or in specific tasks that require eye-limb coordination [46, 47]. As amputation in the lower limb will lead to a lack of movement selection and disorders of movement parameters and coordination, it can be inferred that the GM loss in the PMC following amputation is possibly attributed to long-term use-dependent blockage.

Reduced GM volume in the left primary motor cortex had also been reported in patients with right upper limb amputation [22] but was not found in the current lower limb

amputees. One possible reason for the discrepancy could be the sample heterogeneity between studies. As the upper limb representation is much bigger than the lower limb in the brain [48], the morphological changes due to functional nonuse could be less significant for the patients with lower limb amputation. In line with our reports, one previous study, in which 19 of the 28 patients were amputated at the lower limb, also did not find alterations in the primary motor cortex [20]. In addition, reorganization in the primary somatosensory and motor areas after amputation has been suggested to correlate with PLP [15, 16]. In our study, the amputees with PLP were not included. Therefore, our findings would provide an update on the distinctive patterns of brain plasticity in lower limb amputees without PLP.

In this study, TBSS allowed us to obtain subcortical WM changes across the whole brain in amputees. The PDT approach was used to reconstruct the tracts from the WM skeleton regions characterized by FA decrease in lower limb amputees. This allowed the investigation of abnormal structural connectivity. Our TBSS analysis revealed that right lower limb amputees displayed significant FA reduction in the right superior corona radiata and WM underlying the left PMC. Further fiber tracking generated the transcallosal paths linking the homologous PMC of the bilateral hemispheres. These findings are very consistent with one pioneering DTI study, which reported the reduced integrity in the body of the CC in amputees [23]. It is known that unilateral movement requires sequential processing in bihemispheric motor areas. Using transcranial magnetic stimulation, previous studies found that the PMC modulates the activity of contralateral motor areas during the preparatory period of a voluntary movement with the ipsilateral limb [49, 50]. Such modulation is mediated by interhemispheric inhibition through fibers within the CC [51] and enables healthy adults to perform complex motor tasks without the activation of contralateral muscles [52]. Therefore, the FA reduction within the CC connecting the bilateral PMC may reflect adaptive WM modification following the changes of movement patterns, as the transcallosal inhibition function is disused in unilateral lower limb amputees.

Beyond the left PMC, smaller clusters of cortical thinning in amputees were also noted, mainly in the brain regions constituting visual-to-motor networks, including the bilateral visual cortices, the right temporooccipital junction, left inferior parietal lobule, and orbital frontal cortex. Functional MRI has found that human parietal and temporooccipital cortices constitute the core nodes for cross-modal vision-action representations [38]. Meanwhile, the inferior parietal lobule, particularly in the left hemisphere, contributes to motor attention and is activated in neuroimaging experiments when subjects prepare movements or switch intended movements [53]. Contralateral atrophy in the parietal lobe has also been reported in upper limb amputees [54]. Visual-motor transformation also engages the orbital frontal cortex, which becomes active during response preparation and execution [55]. Further functional/structural connectivity studies confirm that the PMC integrates visual and somatosensory information from the intraparietal area to allow effective exchange and elaboration of information [56]. The connections within the neural networks are plastic and are modified in response to injuries [57, 58], training, and treatments [59]. Previous imaging studies demonstrated that stimulation of afferent input could result in functional reorganization and a corresponding structural expansion of the cortical and subcortical areas [2, 60]. Accordingly, the loss of afferent input following limb amputation should cause "negative" structural alterations with a decrease in GM.

Lower limb amputees also display significant FA reduction in the right IFOF, which is negatively correlated with the time span after amputation. The IFOF connects the inferior frontal lobe to the posterior temporal-occipital regions and provides the main anatomical connections for the ventral (bottom-up) attention system [61], which is specialized for the detection of behaviorally relevant stimuli [62]. Reduction of WM integrity in the IFOF has been reported to be associated with deficits in executive function in patients with chronic trauma [63]. Furthermore, our previous DTI study showed that the right hemispheric IFOF confers an advantage for the executive function of attention [33], which is in line with the well-described rightward dominance of visuospatial processing [64]. Interestingly, the amputees presented time-related microstructural abnormalities of the IFOF in the right rather than the left hemisphere, indicating the degenerative function of visuospatial processing following amputation [65]. Future studies including neuropsychological assessments should be used to investigate the underlying explanations for the associations between brain WM plasticity and visuospatial function in amputees.

The negative findings of GM increase are supported by one MRI study [20] but are incongruent with another [22]. The differences might be due to the status of PLP, prosthesis use, amputation sites, or time span after amputation. Using a smaller sample size, Preißler et al. [22] found that upper limb amputees with slight PLP showed GM increase in regions of the visual stream. They initially hypothesized that it might be a compensatory effect for the lack of sensorimotor feedback and could serve as a protection mechanism against high PLP development [22]. However, in their following study using the same patients, a negative association between prosthesis use and cortical volume in the posterior parietal and occipital lobes, which greatly overlap with the regions with GM loss in our findings, was reported [66]. As prosthesis use has been shown to have a beneficial influence on the prevention of cortical reorganization and PLP [67, 68], and patients rely less often on bottom-up or stimulus-driven control with increasing prosthesis use [66]; we could speculate that the cortical thinning and FA reductions in the ventral visual stream also reflect adaptive brain plastic changes along with the transformation of human abilities and might be beneficial for the prevention of PLP.

Although these findings are robust, some limitations of the present study need to be addressed. First, the relatively small sample size in this study may mask subtle differences between groups, especially in the vertex-based cortical thickness analysis across the whole brain. Therefore, the uncorrected results were reported to minimize type II errors. Second, we only included patients with amputation at the right side. Left lower limb amputation might result

in different morphological and functional changes, especially with respect to the contralateral PMC and the structures in the visual stream. It will be of interest to determine whether the individuals with amputation at the left side will demonstrate the analogous changes at the homologous regions of the other hemisphere. Finally, our explanation of reduced interhemispheric inhibition in amputees is just speculative. Future studies should be performed to confirm the interhemispheric interactions using noninvasive transcranial current or magnetic stimulation.

5. Conclusion

In this study, we combined high-resolution brain structural MRI and DTI to investigate the existence and extent of cortical and WM plasticity in subjects with right lower limb amputation. In summary, we found specific motor and somatosensory plastic changes in amputees without PLP and provided an update on the plasticity of the human brain involving both GM and underlying WM after limb injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guangyao Jiang and Xuntao Yin contributed equally to this paper.

Acknowledgments

This study was supported by the National Natural Science Foundation of China for Young Scholars (no. 81301205) and the Open Project Program of the National Laboratory of Pattern Recognition (NLPR) in China (no. 201306283).

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