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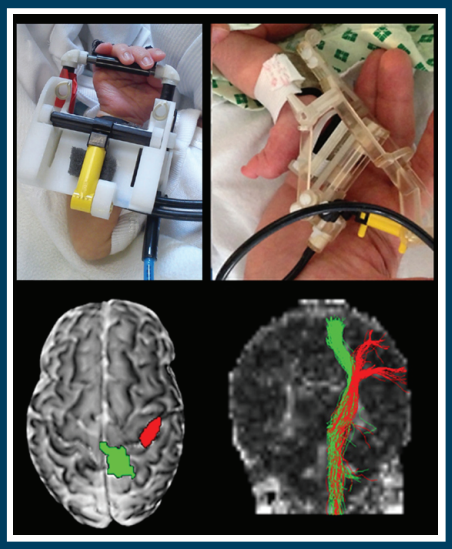

IMPROVING OUTCOMES IN CEREBRAL PALSY WITH EARLY INTERVENTION: NEW TRANSLATIONAL APPROACHES

Topic Editors
Anna Purna Basu and Gavin Clowry

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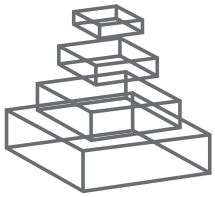

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IMPROVING OUTCOMES IN CEREBRAL PALSY WITH EARLY INTERVENTION: NEW TRANSLATIONAL APPROACHES

Topic Editors:
Dr Anna Purna Basu, Newcastle University and Newcastle upon Tyne Hospitals NHS Foundation Trust, UK
Dr Gavin Clowry, Newcastle University, UK

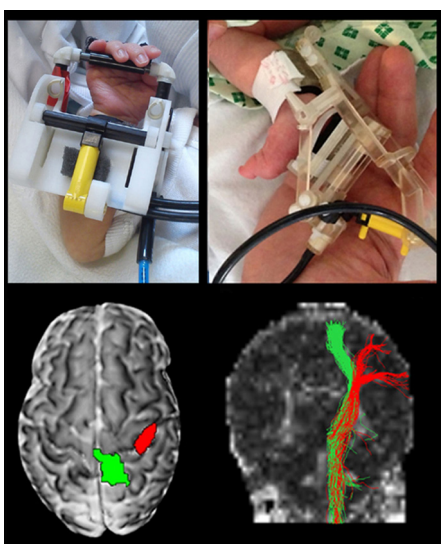

Image taken from Allievi AG, Arichi T, Gordon AL, Burdet E. Technology-aided assessment of sensorimotor function in early infancy. Front Neurol (2014) 5:197. doi:10.3389/fneur.2014.00197

The aim of this Research Topic was to collate articles describing prediction of outcomes of pre- and perinatal lesions leading to cerebral palsy, basic research in animal models and human subjects, and ideas for, and trials of, interventions in the first two years of life.

CP arises from insults to the sensorimotor cortex, subcortical axon tracts and subplate. The aetiology is complex and often multifactorial. The outcome is not simply a loss of voluntary control due to disruption of descending pathways, but also involves abnormal development of reflex and corticospinal circuitry. CP may be viewed as aberrant plasticity in response to a lesion, indeed, abnormalities in movement are subtle at first but develop subsequently. It is misleading to suppose that developmental mechanisms are self-reparative. The challenge is to understand activity-dependent fine tuning of neural circuitry during normal development and to find how to promote desirable plasticity whilst limiting undesirable effects following developmental lesions. However, before proposing interventions, we have to develop our ability to predict the severity of neonatal insults.

We solicited a variety of articles, including long and short reviews, original research and opinion pieces, from both basic scientists and clinicians. Likewise we, as editors, have complementary knowledge and experience in this area. Anna Basu is an academic pediatric neurologist and Gavin Clowry is a developmental neuroscientist.

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EDITORIAL
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Improving outcomes in cerebral palsy with early intervention: new translational approaches

Anna Purna Basu* and Gavin Clowry

Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK
**Correspondence: anna.basu@ncl.ac.uk*

Edited and reviewed by:
Eugene R. Schnitzler, Loyola University Medical Center, USA

Keywords: cerebral palsy, early intervention, translational medical research, diagnosis, outcome prediction

Cerebral palsy (CP) is defined as a group of permanent disorders of the development of movement and posture, causing activity limitation, attributed to non-progressive disturbances occurring in the developing fetal or infant brain (1). Lesions to the sensorimotor cortex, subcortical axon tracts, and subplate are often implicated, with other motor and non-motor areas frequently also affected. The etiology is complex and often multifactorial (2); causes include hypoxia (3), stroke (4), infection (5), trauma, and genetic factors (6). By the end of the second trimester, corticospinal axons have invaded the spinal gray matter and thalamic afferents the upper layers of the neocortex (7, 8). These systems undergo activity-dependent development (9, 10). After early brain injury, descending pathways are disrupted, with abnormal development of reflex and corticospinal circuitry (11, 12). Movement abnormalities are initially subtle but develop subsequently (13, 14). Aberrant post-lesional plasticity undoubtedly contributes to CP. It is misleading to suppose that developmental mechanisms are self-reparative. The challenge is to understand activity-dependent fine-tuning of neural circuitry during *normal* development and promote desirable plasticity while limiting undesirable effects following developmental lesions. However, before proposing interventions, we have to improve our outcome prediction skills.

Cerebral palsy affects 2/1000 live births (15): its prevalence is several times greater than spinal cord injury (SCI) and amyotrophic lateral sclerosis (ALS) (16), which also affect the corticospinal system. However, a Web of Science literature search for 2010–2014 using the phrases "cerebral palsy" (excluding supranuclear palsy), "spinal cord injury," and "amyotrophic lateral sclerosis" returned fewer publications for CP (6653) than SCI (16147) or ALS (8258). For the flagship journals Nature Neuroscience and Neuron, the difference was greater: just one return for CP compared with 39 for SCI and 63 for ALS. Thus, CP, which causes lifelong and often severe disability, is under-researched compared with other conditions that engage neuroscientists and neurologists. We proposed a "Frontiers in Neurology Research Topic" on improving outcomes in CP with early intervention, as a forum to promote CP-related research. We involved authors with expertise ranging from signaling pathways and stem cells through functional imaging and neurophysiology to non-invasive interventions in humans. Articles include long and short reviews, original research, and opinion pieces from basic scientists and clinicians. We achieved our aim in covering prediction of outcomes of pre- and perinatal lesions, basic research in animal models and human subjects, and ideas for, and trials of, early interventions.

Hadders-Algra (17) sets the scene with a comprehensive review summarizing early brain development and discussing the effect of lesions and implications for early diagnosis and intervention. Marcroft et al. (18) review developments in movement recognition technology for classifying spontaneous general movements in high-risk infants. This theme of technology-assisted assessment is further continued by Allievi et al. (19) who focus on the use of instrumented toys and robot-assisted assessment tools with functional MRI so that functional brain activity can be mapped in health and disease even in infancy. Taking a different approach to early detection, Douglas-Escobar et al. (20) explore the potential value of two serum biomarkers of brain damage and neurodevelopmental outcomes in neonates with hypoxic–ischemic encephalopathy (HIE), namely UCH-LI and GFAP.

We received a number of basic research articles relating to early brain injury. Alagappan et al. (21) show that the increase in neural precursor cell growth and proliferation in the subventricular zone after injury depends on insulin-like growth factor receptor signaling as well as EGRF. They discuss how the nature of the culture medium used could have obscured this important finding until now. Again at a signaling pathway level, Frasch (22) considers the role of adenosine monophosphate kinase (AMPK) in inducing adaptive fetal brain shut-down and suppressing pro-inflammatory responses in the context of worsening acidemia during labor. This opinion paper accompanies the article by Xu et al. (23), which explores in an ovine model the complex relationship between preceding chronic fetal hypoxia, acute and worsening acidosis, timing and duration of adaptive brain shut-down, and the degree of brain inflammation. They suggest that EEG monitoring in addition to fetal heart rate monitoring during labor may identify earlier those infants at risk of developing severe acidosis. The ovine model does shed light on the human situation but as ever, extrapolations between species must be done with caution. Clowry et al. (24) address this issue in detail in a review of the suitability of various animal models for testing early intervention approaches in CP.

Moving from physiology to histology and detailed longitudinal neuroimaging, Kostovic et al. (25) characterize white matter lesions in preterm infants in terms of the developmental dynamics of "cellular compartments in the cerebral wall,"

demonstrating how if the precise location and timing of the insult is known, the axonal pathways affected can be predicted. Mackey et al. (26) also use neuroimaging to understand outcome, but in the context of established unilateral CP. In this setting, diffusion-weighted MRI-based fractional anisotropy in the posterior limb of the internal capsule correlates with upper limb functional assessments. They also demonstrate deficits in intracortical and interhemispheric inhibition in those with poor upper limb function.

We also solicited articles on early intervention approaches. Two of these covered cell therapy. Gonzales-Portillo et al. (27) explore the potential for stem cell therapy in neonatal HIE and the outstanding clinical issues to be addressed, while Li et al. (28) discuss umbilical cord blood cell therapies in preterm infants, focusing on white matter injury. The other two articles address non-invasive approaches in infants with unilateral brain damage. Friel et al. (29) review current knowledge of corticospinal tract development including genetic and activity-dependent influences, and describe interventional approaches potentially applicable to hemiplegic CP. Finally, Basu et al. (30) take a clinical standpoint, describing the problems faced in hemiplegic CP, traditional approaches to management and their limitations, and interventions currently under investigation in infants.

We thank everyone who has supported this enterprise by submitting or reviewing manuscripts. We hope this Research Topic will serve its purpose of showcasing some of the fascinating advances in CP research, and raising the profile of this important condition to promote further investigation, ultimately for the benefit of those affected.

ACKNOWLEDGMENTS

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Early diagnosis and early intervention in cerebral palsy

Mijna Hadders-Algra*

Department of Pediatrics – Developmental Neurology, University Medical Center Groningen, University of Groningen, Groningen, Netherlands

Edited by:
Anna Purna Basu, Newcastle University, UK

Reviewed by:
Ivica Kostovic, University of Zagreb, Croatia
Cathy Morgan, Cerebral Palsy Alliance Research Institute, Australia

****Correspondence:***
Mijna Hadders-Algra, Department of Pediatrics – Developmental Neurology, University Medical Center Groningen, University of Groningen, Hanzeplein 1, Groningen 9713 GZ, Netherlands
e-mail: m.hadders-algra@umcg.nl

This paper reviews the opportunities and challenges for early diagnosis and early intervention in cerebral palsy (CP). CP describes a group of disorders of the development of movement and posture, causing activity limitation that is attributed to disturbances that occurred in the fetal or infant brain. Therefore, the paper starts with a summary of relevant information from developmental neuroscience. Most lesions underlying CP occur in the second half of gestation, when developmental activity in the brain reaches its summit. Variations in timing of the damage not only result in different lesions but also in different neuroplastic reactions and different associated neuropathologies. This turns CP into a heterogeneous entity. This may mean that the best early diagnostics and the best intervention methods may differ for various subgroups of children with CP. Next, the paper addresses possibilities for early diagnosis. It discusses the predictive value of neuromotor and neurological exams, neuroimaging techniques, and neurophysiological assessments. Prediction is best when complementary techniques are used in longitudinal series. Possibilities for early prediction of CP differ for infants admitted to neonatal intensive care and other infants. In the former group, best prediction is achieved with the combination of neuroimaging and the assessment of general movements, in the latter group, best prediction is based on carefully documented milestones and neurological assessment. The last part reviews early intervention in infants developing CP. Most knowledge on early intervention is based on studies in high-risk infants without CP. In these infants, early intervention programs promote cognitive development until preschool age; motor development profits less. The few studies on early intervention in infants developing CP suggest that programs that stimulate all aspects of infant development by means of family coaching are most promising. More research is urgently needed.

Keywords: early diagnosis, early intervention, cerebral palsy, neuroplasticity, general movements assessment

INTRODUCTION

Cerebral palsy (CP) is a common neuropediatric disorder with a prevalence of about 2‰ in high-income countries (1) and presumably higher prevalences in lower income countries (2). CP describes a group of disorders of movement and posture. Or, according to the internationally recognized definition of Rosenbaum et al. (3), "cerebral palsy describes a group of developmental disorders of movement and posture, causing activity restrictions or disability that are attributed to disturbances occurring in the fetal or infant brain. The motor impairment may be accompanied by a seizure disorder and by impairment of sensation, cognition, communication, and/or behavior." The definition includes the notion that CP originates during early development, i.e., prenatally or relatively early postnatally. Even though the upper age limit of the postnatal time window is debated (4), CP mostly originates from an event occurring before the age of 6 months corrected age (CA).

The definition of CP highlights the diversity of neural impairments involved in CP, while simultaneously underlining the implications of the impairments for activities and participation. Nowadays, the major goal of rehabilitation services is to optimize home and community participation (5), implying that clinical management comprises all aspects of the framework of the international classification of functioning, disability and health, child and youth version [ICF-CY (6)]. As a result, clinicians working in the field of neuropediatrics and pediatric rehabilitation need to understand topics varying from neurodevelopmental mechanisms to family function. The aim of the present paper is to briefly review and critically discuss (a) prenatal and early postnatal brain development, the effect of an early lesion of the brain, and the consequences of neurodevelopmental principles for early diagnosis and early intervention in CP, (b) tools for early diagnosis, and (c) early intervention.

PRENATAL AND EARLY POSTNATAL BRAIN DEVELOPMENT

INTRICATE PROCESSES OF BRAIN DEVELOPMENT

The development of the human brain is an intricate and long-lasting process. This is particularly true for the development of the neocortical circuitries; it takes about four decades time before they have established their "adult" configuration (7). Here, I will primarily discuss the developmental processes occurring in the prenatal and early postnatal period and I will focus on the neocortex and cerebellum, the structures where the vast majority of human neurons can be found (8). First, neocortical development is described. This description also serves to illustrate the complex and only partially understood developmental processes in the brain. Next, cerebellar development is discussed.

Development of the neocortex

Neocortical development starts during the early phases of gestation with the proliferation of neurons. The majority of telencephalic neurons are produced in the first half of gestation in the germinal layers near the ventricles (9, 10). Young neuroblasts move from their place of origin to their final place of destination in the more superficially located cortical plate (9, 11). Neural migration is guided by the shafts of transient radial glial cells (10). However, initially developmental focus does not center on the cortical plate, but on a temporary structure, i.e., the subplate [Ref. (8), **Figure 1**]. The subplate is situated between the cortical plate and the intermediate zone, i.e., the future white matter (12). It contains a variety of neurons, most of which are glutamatergic (13). The subplate is thickest around 29 weeks postmenstrual age (PMA), when it is about four times thicker than the cortical plate (8). Thereafter, the subplate gradually disappears during the perinatal and early postnatal period, although it remains present below the associative cortices up to 6 months post-term (8).

Neurons start to differentiate during migration. Neuronal differentiation includes the formation of dendrites and axons, the production of neurotransmitters and synapses, and the elaboration of the intracellular signaling machinery and complex neural membranes (15, 16). The subplate, which became increasingly important during phylogeny (17), plays an important role in the processes of differentiation and cortical organization (8, 18). It is the major site of neocortical synaptogenesis. It also serves as a waiting and guidance compartment for growing cortical afferents, in particular, thalamocortical and corticocortical fibers. The cortical afferents "wait" for several months in the subplate before relocating from 28 weeks PMA onward into their final target, the cortical plate (8, 19). Evidence suggests that the ingrowing thalamocortical fibers meet the corticofugal projections of early-born preplate neurons. In other words, early corticofugal projections form "hand-shaking" scaffolds for the ingrowing thalamocortical fibers (20). During their fetal presence, the diverse and transient circuitries of the subplate are a prominent site of synaptic interaction; the subplate neurons produce spontaneous activity, and process the sensory information of the thalomocortical fibers (8).

The processes of neural differentiation and cortical organization are particularly active in the few months prior to birth and the first postnatal months. Developmental processes in the subplate, i.e., in the cortical–subcortical interface, continue to play a prominent role in cortical organization. During this period, the human cortex is characterized by the co-existence of two separate but interconnected cortical circuitries; the transient fetal circuitries centered in the subplate and the immature, but progressively developing permanent circuitry centered at the cortical plate (8). The duration of the "double circuitry" phase differs for the various regions in the cortex. For instance, the final phase of permanent cortical circuitry is reached around 3 months postnatally in the primary motor, sensory, and visual cortices, but first around the age of 1 year in the associative prefrontal cortex (18).

Besides neural cells, glial cells are generated. The peak of glial cell production occurs in the second half of gestation. Glia cell production includes the generation of oligodendrocytes, the cells involved in axonal myelination. Oligodendrocyte development reaches its peak between 28 and 40 weeks PMA (13). Myelination takes place especially between the second trimester of gestation and the end of the first postnatal year. It occurs earlier in sensory pathways than in motor ones, and earlier in projection fibers than in associative fibers (21). Beyond infancy, myelination continues until the age of about 40 years when the last intracortical, in particular, the long-fronto-temporal connections such as the cingulum, complete myelination (22).

Brain development does not only consist of the creation of components but also of an elimination of elements. About half of the created neurons die off by means of apoptosis. Apoptosis is brought about by interaction between endogenous programed processes and chemical and electrical signals induced by experience (23). In the neocortex, apoptosis occurs, in particular, between 28 weeks PMA and term age (24). Not only neurons are removed but also axons and synapses are eliminated. A well-known example is the pruning and tuning of the corticospinal

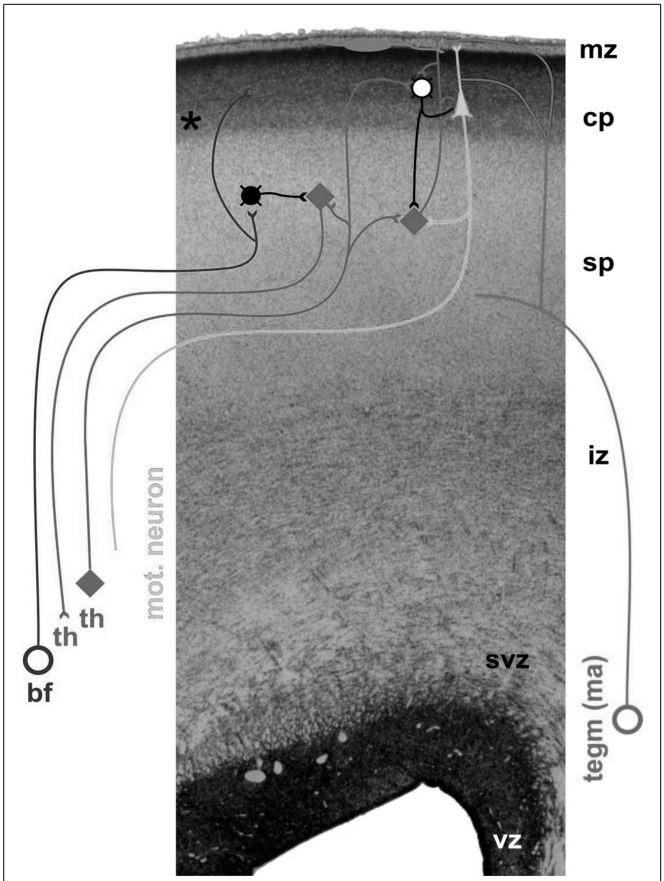

FIGURE 1 | Cross-section through the cortex of a fetus of 24 PMA. The following layers can be distinguished, from the inside (bottom) to the outer surface (top): vz, the ventricular zone, which produces neurons; svz, the subventricular zone, which possibly is phylogenetically younger than the ventricular zone, and which produces neurons and glial cells (14); iz, the intermediate zone, i.e., the future white matter; sp, the subplate, which at this stage is very thick and harbors the transient fetal circuitry; cp, the cortical plate; mz, the marginal zone. Ingrowing afferents come from the basal forebrain (bf), thalamus (th), and monoaminergic brain stem nuclei (tegm ma). Figure by curtsy of Dr. Ivica Kostovic, University of Zagreb.

tract: during the last trimester of gestation and continuing in the first two postnatal years the initially bilateral corticospinal projections in the spinal cord are reorganized into a mainly contralateral fiber system (25). This reorganization is activity driven and use dependent, as is illustrated by the effect of an early unilateral lesion of the brain. The latter results in asymmetrical activation of the spinal cord, inducing a preferential strengthening of the activity from the ipsilateral projections from the contralesional hemisphere in comparison to the contralateral projections from the ipsi-lesional hemisphere (25, 26).

The elimination of synapses in the brain starts already during early development, but in the neocortex this process becomes especially prominent between the onset of puberty and early adulthood. As a result, developmental remodeling of cortical neuronal circuitries continues well into the third decade of life (27).

Development of the cerebellum

Both the classical studies of John Dobbing (28, 29) and modern imaging studies (30) revealed that the cerebellum develops at high speed between 24 and 40 weeks PMA. Cerebellar volume increases with a factor 3 and cerebellar surface – during the formation of the characteristic cerebellar "folia" – with a factor 30 (31). In 2009, Joseph Volpe excellently reviewed the developmental processes in the cerebellum (31). Below, I summarize his review.

In the cerebellum, two proliferative zones can be distinguished: (a) the ventricular zone, which gives rise – by radial migration – to the deep cerebellar nuclei and the Purkinje cells, and (b) the rhombic lip, which gives rise – by tangential migration – to the external granular layer (**Figure 2**). The external granular layer is a transient structure that reaches its peak thickness between 20 and 30 weeks PMA. At that time, the cells of this layer (the granule cells) start to migrate inward – guided by Bergmann glial fibers – through the molecular layer with Purkinje cells, to their destination in the internal granular layer. During the inward migration, the granule cells form horizontal parallel fibers that contact the Purkinje cells. When the granule cells have arrived in the internal granular layer they soon receive input from the mossy fibers from the pons. Between 30 and 40 weeks PMA, the external granular layer is heavily involved in cell proliferation. It results in the previously mentioned fabulous expansion of the cerebellar surface. Meanwhile, the inward migration of the granule cells to the internal granular layer continues. In the first postnatal year, the external granular layer decreases in size and activity. Simultaneously, the internal granular and molecular layer increase in size. The latter is especially due to the elaboration of granule cell axons (parallel fibers) and Purkinje cell dendrites.

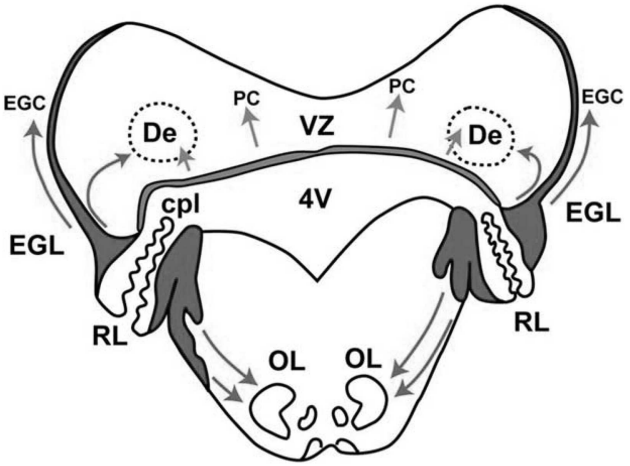

FIGURE 2 | Schematic representation of the two proliferative zones in the cerebellum around 14 weeks PMA, the dorsomedial ventricular zone (VZ), and the dorsolateral rhombic lip (RL). The VZ gives rise to the interneurons of the deep cerebellar nuclei, such as the dentate (De) and to Purkinje cells (PC). Migration occurs radially. The RL has two portions divided by the choroid plexus (cpl) of the 4th ventricle (4V). The upper portion gives rise to the granule precursor cells (EGC) of the external granular layer (EGL) – the cells initially migrate tangentially over the surface of the cerebellum. The tangential migration is later followed by an inward migration to the internal granular layer. The lower portion of the RL gives rise to neurons in the pons, including those of the inferior olive (OL). The arrows indicate the directions of migration. With permission from Dr. Joseph Volpe (31).

EFFECT OF AN EARLY LESION OF THE BRAIN

Over the years, animal data have demonstrated that the effect of a lesion of the developing brain depends on the point in time at which the lesion occurred. Originally, it was thought that "the younger the age at insult, the better the outcome" [the so-called Kennard-principle (32)]. But gradually it became clear that this is not always true (33). Many factors determine the consequences of a lesion of the developing brain: the age at insult, the site, and the size of the lesion, its unilateral or bilateral nature, animal species, sex, exposure to chemical substances prior to and after the insult, and environmentally induced experience. Rodent studies indicated that, in particular, two types of environmental experience are associated with improved outcome: being raised in a complex environment and tactile stimulation at early age (33, 34). Animals with an early lesion of the brain who are raised in a complex environment, including attractive toys and peers, have a significantly better motor and cognitive outcome than lesioned animals brought up in a standard, "boring" laboratory environment. The improved functional outcome is associated with increases in brain weight, cortical thickness, and dendritic length. It has been suggested that part of the effect of the complex environment is mediated by increased maternal care in the form of licking and grooming, i.e., early tactile stimulation. Indeed, other studies revealed that tactile stimulation of pups, who acquired an early lesion of the brain, is associated with improved motor and cognitive outcome and increased dendritic spine density, changes that presumably are mediated by increased levels of neurotrophic factors (33). The complex picture emerging from the animal studies is that, each age, each neural system, each species, and each sex has specific vulnerabilities and resources of resilience to cope with the effects of an early lesion. Nevertheless, within the complexity three general principles may be distinguished: (a) bilateral lesions are associated with a lower potential for functional plasticity and with worse outcome than unilateral lesions; (b) large (unilateral) lesions are associated with less recovery and worse functional outcome than small (unilateral) lesions; (c) cognitive functions show a better recovery than motor functions (33).

Retrospective magnetic resonance imaging (MRI) studies in children with CP demonstrated that the most common brain lesion in these children is damage of the periventricular matter. A recent review of population-based studies carried out in western industrialized countries, revealed that a lesion of the periventricular white matter is present in 19–45% of children with CP (35). Other relatively frequent lesions are gray matter injury, including lesions of the cortical gray matter, the basal ganglia, and the thalamus (21%), malformations (11%), and focal cortical infarcts (10%) (35). Note that in about 15% of children with CP structural MRI scans do not show abnormalities (35, 36). The varied MRI findings illustrate the neurodevelopmental heterogeneity of CP. However, the findings do not inform us about the neural mechanisms operating when the brain acquires a specific lesion at a certain early age. These mechanisms may involve plastic, restorative adaptations, but they also may result in deleterious changes.

Below, I summarize the neurodevelopmental sequalae of two major categories of brain lesions: damage of the periventricular white matter and unilateral lesions of the brain.

Damage of the periventricular white matter

Lesions of the periventricular white matter mostly originate between the ages of 24 and 34 weeks PMA. Prospective imaging studies on the developmental sequelae of damage of the periventricular white matter indicated that focal necrotic lesions [cystic periventricular leukomalacia (PVL)] are associated with a high risk for CP [>80% (37, 38)]. The risk for CP is higher in posterior than in anterior lesions (39). In addition, the severity of CP following PVL depends on the severity of the cystic lesion: focal cysts generally give rise to bilateral CP with a diplegic distribution and more extensive cysts result in bilateral CP with a quadriplegic distribution (39). In fact, the cystic lesions are the tip of the iceberg of the pathology in the periventricular white matter, as the cystic lesions are surrounded by diffuse astrogliosis and microgliosis in the white matter (13, 40). Actually, in modern neonatal intensive care units (NICUs) cystic PVL accounts for only a minority of the lesions of the periventricular white matter. Much more common is the "non-cystic PVL," which consists of diffusely distributed, small lesions of the periventricular white matter (13). The specific characteristics of non-cystic PVL in neonatal imaging are debated [ultrasound, periventricular echodensities (38); MRI, punctate lesions and diffuse excessive high-signal intensity (39)]. Notwithstanding the variation in criteria for non-cystic PVL, the data indicate that non-cystic PVL is also associated with CP, be it with lower risk rates than cystic PVL (38).

Brain pathology in children with PVL is, however, not restricted to the periventricular white matter of both hemispheres. PVL is accompanied by a half to three-quarter reduction in the number of the most prevalent type of neurons in the subplate, i.e., the polymorphic non-pyramidal and inverted pyramidal neurons. This reduction does not only occur at the site of the lesion but also in remote areas (40). PVL also is associated with a bilateral decrease in cerebellar volume (31), an altered arborization of the noradrenergic fibers in the cerebellar circuitries (41), and – in a substantial proportion of children – with neuronal loss in the thalamus and basal ganglia (13). The associated pathologies may be the result of the hypoxic-ischemic and inflammatory events that caused the PVL, but they may also be the result of diaschisis, i.e., the loss of function in a neurally connected region (31). More recently, it has been suggested that the injury processes that cause PVL, including persistent inflammation and epigenetic changes, may persist for many months or even years (42). The latter implies that the well-known "growing into a deficit" principle manifested during the development of a child with CP (33, 43) may not only be caused by the age-related development of increasingly complex cerebral functions and therewith the expression of impairments in these functions but may also be the result of progressive damage. The widespread encephalopathy of PVL explains why PVL often results in bilateral CP that is often associated with cognitive impairments (44). Unfortunately, the widespread bilateral brain damage and its motor and cognitive sequelae limit the possibilities of early intervention to induce beneficial plastic changes in the brain (33).

Unilateral lesions of the brain

Basically, two types of unilateral lesions of the brain can be distinguished: (a) unilateral periventricular hemorrhagic infarction, occurring in preterm infants of 24–34 weeks PMA, and involving the periventricular white matter [further referred to as "preterm" lesions (45–47)], and (b) focal cortical–subcortical infarction, occurring around term age, and usually affecting the area of the medial cerebral artery (further referred to as "term" lesions). The term lesion in general does not involve the periventricular white matter (47, 48). These two lesions may be regarded as the two ends of the developmental spectrum of perinatally acquired unilateral lesions of the brain. In clinical life also mixed patterns exist. In addition, the lesions may also occur bilaterally – the ratio between unilateral and bilateral forms being 3:1 (46, 48). Below, I will discuss the sequelae of the unilateral lesions in children who develop CP. It should be realized, however, that about 25–50% of the infants with a perinatally acquired unilateral lesion of the brain does not develop CP (48–51). The children with a unilateral brain lesion, who do develop CP, mostly present with unilateral CP, but some infants develop bilateral CP (52).

Staudt and co-workers demonstrated in children with unilateral CP that the plastic events in response to a unilateral lesion do not only vary with the timing of the lesion but also with the neural system involved (47, 53). In the motor system, the reorganization may involve persistence of the typically transiently present ipsilateral corticospinal projections. The chance that ipsilateral projections persist increases with decreasing gestational age. As a result, the function of the paretic hand in children with unilateral CP resulting from a preterm lesion often involves ipsilateral corticospinal activity, and the function of the paretic hand in children with unilateral CP resulting from a term lesion generally involves contralateral corticospinal activity (25, 47). However, many mixed patterns exist. In general, the presence of more ipsilateral projections is associated with worse bimanual function (54). In part, the worse bimanual function may be due to hindering "mirror movements," as motor control of both hands is mediated by the same corticospinal system (25, 47).

For the sensory system, the effect of a preterm unilateral lesion differs from that occurring in the motor system. In the sensory system, reorganization does not involve structures on the unaffected

side of the brain. It is mediated by structures on the lesioned side (47, 53, 55). This reorganization is related to the way in which the sensory system develops at early preterm age. At that time, the ascending thalamocortical somatosensory projections have not yet reached the cortex, allowing the ingrowing axons to take a detour, and bypass the lesion in order to reach the cortex. This axonal plasticity is associated with good or only minimally reduced somatosensory function. In term unilateral lesions, such reorganization is no longer available. Consequently, lesions often result in severe somatosensory deficits (47).

The differential reorganization between motor and sensory functions imply that sensorimotor control in children with unilateral CP resulting from a preterm lesion differs from that resulting from a term lesion (**Figure 3**). In children with a typical preterm lesion control is dissociated; somatosensory information of the paretic hand is processed by the lesioned, contralateral hemisphere, but motor commands for the paretic hand are generated in the non-lesioned, ipsilateral hemisphere (53, 56). This neurophysiological make-up is usually associated with a relatively intact processing of sensory information in combination with moderate motor control. In children with a term lesion, somatosensory processing and motor control of the paretic hand both occur in the lesioned, contralateral hemisphere. Manual ability in these children varies considerably, and presumably is largely affected by the substantial somatosensory deficits (47, 57).

The language system is characterized by yet another form of plasticity (47, 56). In the majority of human adults, language develops predominantly in the left hemisphere. In infants, who perinatally acquire a lesion of the left hemisphere, the language system may move entirely to the homotopic area in the right hemisphere, with no or little loss of function (47). This happens more often in infants with a term lesion than in those with a preterm lesion (58).

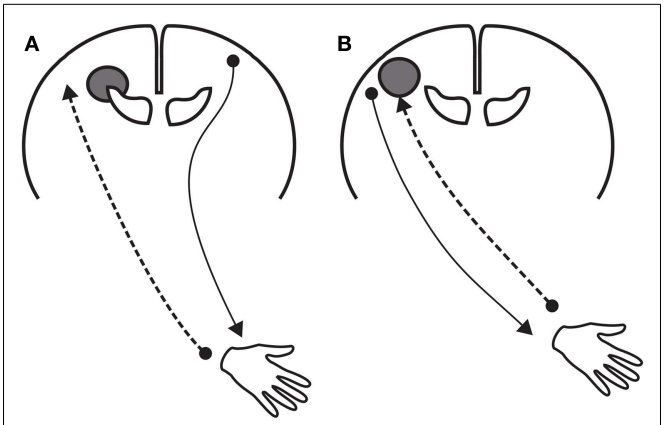

FIGURE 3 | Schematic representations of the reorganization of motor and sensory function after a unilateral lesion of the brain at early age. **(A)** Reorganization after a "preterm" unilateral lesion. The lesion involves the periventricular white matter. The reorganization includes (a) the persistence of ipsilateral corticospinal projections to the paretic hand originating in the contralesional hemisphere and (b) axonal plasticity of the thalamocortical afferents bypassing the lesion in the ipsi-lesional hemisphere. **(B)** Reorganization after a "term" unilateral lesion, which usually does not include the periventricular white matter. Motor and sensory functions of the paretic hand are organized in the lesioned hemisphere.

IMPLICATIONS FOR EARLY DIAGNOSTICS AND EARLY INTERVENTION

The population-based MRI review of Reid and colleagues (35) indicated that in western industrialized countries 50–75% of children with CP acquire their lesion between 24 weeks PMA and term age. This is the period during which brain development is characterized by a high rate of widespread and complex developmental processes. The rapid changes over time do not only induce age-specific vulnerabilities of the brain, e.g., lesions in the periventricular white matter at early preterm age and lesions in the cortical–subcortical areas around term age, but also induce age-related plasticity. As a consequence, early neurological dysfunction after preterm lesions may be expressed in a different way than that after term lesions. For instance, during the first months post-term infants with a preterm lesion of the brain developing CP may present with more or less typical muscle tone and reflexes in combination with an abnormal quality of general movements, whereas infants with a term lesion of the brain developing CP usually present with dysfunction in all aspects of neurological function, i.e., in muscle tone, reflexes, and the quality of general movements (59–61).

Relatively little data are available on the prevalence and the etiology of CP in low- and middle-income countries (LMIC). The limited LMIC-data available suggest that CP in these countries is less often caused by complications associated with preterm birth than in western industrialized countries, and more often by asphyxia and hyperbilirubinemia at term, and by postnatal infections, such as meningitis (62–64). This means that the presentation of CP in LMIC differs from that in western industrialized countries. The differences involve different etiological mechanisms, a different timing of the lesion and different plastic changes of the brain. This may mean that knowledge on early diagnostics and early intervention coming from western industrialized countries may not immediately be generalized to LMIC.

Early diagnostics and early intervention after a perinatal lesion of the brain occur especially in the preterm period and during the first year post-term. During this period, the double neocortical circuitry, in which the subplate and cortical plate circuitries co-exist, is gradually substituted by the single circuitry of the developing networks in the cortical plate. Also, the cerebellum shows dramatic developmental changes during this period. For diagnostics, these large neurobiological changes have major consequences. First, the fact that a child has an age-specific nervous system invokes the need of age-specific assessments, that is, evaluation techniques, which are adapted to the age-specific characteristics of the nervous system. Examples are age-specific neurological, motor, and cognitive assessments. Apart from the need of age-specific instruments, assessments are in need of age-specific norms. This is not only true for functional assessments, such as neurological exams and cognitive tests, but also for imaging techniques and physiological assessments. Second, the age-dependent characteristics of the nervous system affect the way in which neural dysfunction is expressed. Neurological dysfunction in adults is expressed by means of specific and localized signs, e.g., by means of the specific syndrome of a spastic hemiplegia in case of stroke. In contrast,

neurological dysfunction in young infants is expressed by means of generalized and unspecific dysfunction. For instance, a preterm infant with a left-sided infarction may respond with generalized hypotonia, generalized hypertonia, hypokinesia, a hyperexcitability syndrome, or with abnormal general movements (43, 65). In infants developing CP, the early unspecific neurological dysfunction gradually develops into the specific syndrome of CP. This development may take 1–5 years, but in most children the diagnosis can be established by the age of 18–24 months (4). Third, the marked developmental changes of the brain have important implications for the prediction of developmental disorders at early age. The plastic changes may induce a disappearance of dysfunctions present at early age – infants grow out of their deficit. The reverse is also possible: children may be virtually free from signs of dysfunction at early age, but grow into a functional deficit with increasing age due to the age-related increase in complexity of neural functions (66).

The amazing developmental changes of the brain between preterm age and the age of 1 year post-term offer opportunities for early intervention. Animal studies indicated that intervention has the largest effect when applied during the period when dendrites and synapses are produced at a high rate. The period during which the double cortical circuitry configuration wanes offers therefore large opportunities for early intervention. On the other hand, it should be realized that the early lesions themselves often are associated with pathological processes elsewhere in the nervous system, either as a remote consequence of the primary lesion or as a concomitant effect of the harmful events that caused the primary lesion. In addition, the prenatal and perinatal complications, resulting in the lesion of the brain may also imply the presence of a prolonged period of stress and pain for the young infant. Stress during early life is known to have lifelong consequences, as it induces permanent changes in the brain, especially in the mono-aminergic systems, such as the dopaminergic system (67, 68). Alterations in the dopaminergic system are associated with impaired motor learning (69). Indeed, it has been demonstrated that preterm infants with and without lesions on the ultrasound scan of the brain have deficits in motor learning. They have more difficulties than typically developing term infants to build internal reference frames of body configuration on the basis of daily experience. As a result, they rather rely on simple feedback motor control strategies than on feedforward motor control (70). Unfortunately, the presence of widespread neural impairment in the brain reduces the potential for plastic changes of the young nervous system, as animal experiments indicated that the chance of beneficial effects of intervention decreases with the extent of brain pathology (33, 34).

EARLY DIAGNOSTICS IN CP

The preceding paragraph stressed that the developmental changes of the brain hamper prediction of CP at early age. This does, however, not imply that we are totally at loss with prediction. Prediction improves when we use multiple tools, such as neuroimaging, neurological and neuromotor exams, and neurophysiological assessments (71–73). Prediction also improves substantially when longitudinal series of assessments are used – developmental trajectories predict developmental outcome best (74–76). Knowledge on the predictive value of single assessments with specific diagnostic instruments is generally based on selective groups of high-risk infants in western industrialized countries (77). This means that the findings may neither be generalized to populations of high-risk infants in LMIC settings nor to general populations across the world, which mostly consist of low-risk infants. In this respect, it is also important to note that the Surveillance of CP in Europe study revealed that 70% of children with CP had been admitted to a special care infant unit after birth (78). Recall too that the retrospective imaging study of Reid et al. (35) indicated that 50–75% of brain lesions occur between 24 weeks PMA and term age. Both types of data suggest that 25–50% of children with CP will not show signs suspect for CP during the newborn period, and thus, will not receive neonatal monitoring to predict developmental outcome. In the following sections, I will address the assessment methods that are most frequently used in the early prediction of CP: (a) neurological and neuromotor assessments, (b) neuroimaging, and (c) neurophysiological tests.

NEUROLOGICAL AND NEUROMOTOR ASSESSMENTS

Neurological assessments are frequently used to monitor development of high-risk infants. The best known methods are the Dubowitz assessment for neonates (79) and its adaptation for older infants, the Hammersmith infant neurological examination [HINE (80)], the Prechtl assessment for newborns (65) and its adaptation for older infants, the Touwen infant neurological examination [TINE (75, 81)], and the assessment according to Amiel-Tison (82). Predictive validity of these assessments is generally good (82), with an estimated sensitivity and specificity for CP of 88–92%, respectively (77).

The best known neuromotor assessment is the general movement assessment (GMA). General movements are the most frequently used movements from early fetal age until 3–4 months post-term (83). The quality of these movements provides information about the integrity of the brain, possibly especially about the connectivity in the periventricular white matter (84). Typical general movements are characterized particularly by variation and complexity; in abnormal general movements these characteristics are reduced or absent (83, 84). Prediction of CP with the GMA is excellent when based on longitudinal series of assessments (84). When a single assessment is used, prediction is best when GMA is carried around 3 months post-term [median sensitivity 98% (range 50–100%), specificity 94% (range 35–100%) (77, 85, 86)]. Other motor assessments used to predict CP in infancy are the motor assessment of infancy [MAI (87)], the test of infant motor performance [TIMP (88)], the Alberta infant motor scale [AIMS (89)], the infant motor profile [IMP (90)], and – less frequently – the psychomotor index of the Bayley scales of development (91). However, calculation of predictive values for CP is precluded due to the limited data on prediction available, or due to the fact that studies evaluating the predictive properties used more global "abnormality" outcomes (77, 85, 92).

The neurological and neuromotor assessments are relatively cheap instruments, and therefore, may be applied in many settings across the world. Another instrument that may be used in low-risk populations and in settings with limited resources is the developmental assessment of young children [DAYC (76)]. The

DAYC has a complementary approach: parental information on motor milestones serves as the starting point for a quick testing of the limits of the infant's skills. The retrospective study of Maitre et al. (76) demonstrated that a decrease in DAYC-scores between 6 and 12 month of age was highly predictive of CP.

NEUROIMAGING

Neurological condition in infants admitted to a NICU is virtually always evaluated by imaging of the brain, in particular, by cranial ultrasound (cUS) and MRI. Neonatal cUS is especially applied in preterm infants. It readily visualizes large lesions of the periventricular white matter, such as periventricular hemorrhagic infarction and cystic PVL. For proper prediction of outcome, it is recommended to make series of cUS, i.e., sequential cUS during the first 4–6 weeks after birth and an additional one at 36–40 weeks PMA, as it takes 2–5 weeks for cysts to develop (38). Meta-analysis of six studies including over 2400 preterm infants on the power of neonatal cUS to predict CP indicated an estimated sensitivity of 74% and an estimated specificity of 92% (77). cUS may also assist prediction of the type and severity of CP (93). Unilateral infarctions are associated with unilateral spastic CP (but recall that this is not a one-to-one relationship!). Deep gray matter lesions are associated with dyskinetic CP and severe impairment (93). In case of PVL, cUS also serves the prediction of the ability to walk independently at the age of 2 years: children with grades I and II PVL usually are able to walk at age 2, whereas <10% of children with grade III (extensive PVL) are able to do so (38).

Magnetic resonance imaging is the preferred imaging technique in (near) term infants with hypoxic-ischemic neonatal encephalopathy. It is also increasingly used in preterm infants. In the latter group, the best age for MRI is term equivalent age (TEA), i.e., 40–42 weeks PMA. Scans at earlier ages run the risk of missing relevant damage of the posterior limb of the internal capsule (38). Limited data on the precise prediction of CP with neonatal MRI are available. However, existing data suggest that high sensitivity and high specificity (38, 77). Currently, also more sophisticated MRI techniques are applied, such as diffusion-weighted imaging [to visualize hypoxic-ischemic brain lesions (94, 95)], diffusion tensor imaging [DTI, to visualize the microstructure of the white matter (96)], and magnetic resonance spectroscopy imaging [for detailed information on local brain metabolism (95)]. These instruments are promising, in particular, DTI of the posterior limb of the internal capsule (96), but details on prediction of CP are very limited.

NEUROPHYSIOLOGICAL TESTS

Neurophysiological tests are especially used in term infants with neonatal encephalopathy. The recent systematic review of Laerhoven et al. (95), which addressed the predictive value of various tests in infants who mostly suffered from moderate to severe neonatal encephalopathy, indicated that the amplitude-integrated electroencephalogram (aEEG) and traditional electroencephalogram (EEG) predicted outcome well. Abnormal outcome was, however, defined as either death or having a moderate to severe disability. More recently, the aEEG is also applied in preterm infants. Also, in this group aEEG predicts outcome in terms of death and disability well (97, 98). However, due to the combined outcome measure of death and disability – this implies that data on the value of aEEG and EEG to predict CP are currently lacking (99).

Additional neurophysiological methods, which are used to predict developmental outcome in high-risk infants are the visual evoked potential (VEP) and somatosensory evoked potential (SEP). SEP is recorded as a reaction to stimulation of the median nerve or the posterior tibial nerve. The review of Laerhoven et al. (95) indicated that both SEP and VEP may help to predict death or disability in infants with moderate to severe neonatal encephalopathy. However, the predictive values of SEP and VEP are less than those of aEEG and EEG. Little is known on the value of neonatal SEP and VEP to predict CP. The relatively small studies of Suppiej et al. [in infants with neonatal encephalopathy (100)] and Pike and Marlow [in preterm infants (101)] suggested that SEP presumably predicts abnormal neuromotor outcome better than VEP.

EARLY INTERVENTION IN CP

The effect of early intervention has been studied predominantly in infants at high risk for developmental disorders, i.e., in groups of preterm infants only (102), or in mixed groups of high-risk infants (103). The large majority of the infants studied did, however, not develop CP. This implies that our knowledge on early intervention in infants who do develop CP is limited. I will first address the effect of early intervention in high-risk infants. Next, I will discuss the data available on early intervention in infants who later are diagnosed with CP.

EARLY INTERVENTION IN HIGH-RISK INFANTS

In preterm infants intervention may start prior to term age. The limited evidence available suggests that the newborn individualized developmental care and assessment program (NIDCAP) and infant massage (103, 104) are associated with a short-term beneficial effect on brain development. However, the evidence on long-term effects of these interventions is inconclusive. On the effect of early intervention in preterm infants after term age more information is available. A meta-analysis (105) and a systematic review (102) indicated that early intervention by means of general developmental programs is associated with a positive effect on cognitive development until the age of 3 years. Whether or not the effect persists beyond this age is not clear, as few studies followed the children at school age or in adolescence. The systematic review of Spittle et al. (102) suggests that the effect of early intervention disappears after preschool age. However, follow-up of the infant behavioral assessment and intervention program (IBAIP) and the infant health and development program (IHDP) indicates that some effects of early intervention may sustain beyond preschool age (106, 107). The IBAIP program applied in very low-birth weight infants was associated with a minor advantage in performance intelligence quotient, visuomotor integration, and the ball task "aiming and catching" at the age of 5.5 years (106). The IHDP program, which was applied in low-birth weight infants, was associated with higher scores on vocabulary and mathematics tests and with less risk behavior at 18 years of age. However, these effects were found only in the subgroup of participants with a birth weight of 2000–2500 g and not in those with a lower birth weight (107). The authors suggested that adverse biological factors may offset the beneficial effect of intervention.

The early intervention studies also showed that in general the effect of developmental programs on motor development is small and does not persist beyond infancy (102, 105). Wallander et al. (108) applied the concept of early developmental intervention in asphyxiated infants in the LMIC setting. The results of this study were similar to those of the early intervention studies in preterm infants in more affluent settings: intervention promoted development until the age of 3 years, and the effect on cognitive development was larger than that on motor development. The systematic review of Blauw-Hospers and Hadders-Algra (103) paid special attention to the contents of the various early intervention programs. The review underlined two points. First, no evidence is available for a beneficial effect of early intervention by means of neurodevelopmental treatment (NDT) or Vojta therapy. Second, the general developmental programs that are associated with a positive effect on developmental outcome are very heterogeneous. This implies that our knowledge on the effective elements of intervention is limited.

Early intervention in general comprises in addition to the therapeutic developmental interventions targeting the infant, some form of parental support, including psychosocial support and parent education. As a result, general developmental programs are also associated with a reduction of maternal anxiety and depression, improved maternal self-efficacy, and – presumably – less maternal stress (109). Possibly, the effect of the programs on the mother is one of the mediators of the effect of early intervention on the infant's development. However, the way in which parents are involved in early intervention differs considerably. Traditionally, parents have been assigned the role of co-therapist. But gradually, awareness of family autonomy arose, leaving room for individual parenting and educational styles in early intervention (110, 111). The concept of family coaching as opposed to parent training emerged (111). Family coaching in early intervention implies that families set the goals for intervention and that the coach provides – by means of an open dialog – hints and suggestions how the goals may be achieved during daily routines, such as feeding and bathing (110–112). A recent study in high-risk infants indicated that family coaching during early infancy was associated with improved motor development and functional mobility at 18 months CA (113, 114). This suggests that family coaching may be one of the potentially effective factors in family centered care.

EARLY INTERVENTION IN INFANTS DEVELOPING CP

Very little information is available on the effect of early intervention in the subset of high-risk infants, who later are diagnosed with CP. When I refer to this subgroup, I will use the expression "infants developing CP" or "infants who develop CP." However, note that this label can be assigned only retrospectively. Four studies addressed the effect of general early intervention programs in infants with brain lesions on cUS (115–117) or in infants who developed CP (113, 114). Nelson et al. studied in 37 infants, the effect of auditory, tactile, visual, and vestibular stimulation provided from preterm age to 2 months CA. The intervention was not associated with a difference in motor and cognitive outcome at 12 months CA assessed with the Bayley scales of infant development (115). Follow-up data were, however, only available in 27 infants, meaning that the study suffered from the risk of selection bias due to substantial attrition and that the study was underpowered. Ohgi et al. assessed in 23 infants, the effect of a neonatal behavioral intervention starting in the preterm period and continued until 6 months CA. The behavioral intervention was associated with improved behavioral state regulation at 4 weeks CA, but no effect of intervention could be demonstrated on motor and cognitive development at 6 months CA measured with the Bayley scales of infant development (116). It should be noted, however, that the study was underpowered. Weindling et al. compared in 87 infants of whom 45 developed CP, the effect of infant physical therapy based on NDT to standard care. Motor and cognitive outcome measured with Griffiths developmental assessment of both groups was similar at 3 years of age (117). Also, this study lacked the power to demonstrate statistically significant differences. In the Netherlands, the effect of the family centered program COPCA [coping with and caring for infants with special needs (110, 111)] applied between 3 and 6 months CA was evaluated in 10 infants who developed CP. Developmental outcome at 18 months CA of the group who had received COPCA intervention did not differ from the group that had received traditional infant physical therapy. Also, this study was underpowered. In addition, quantitative video-analysis of the contents of the interventions demonstrated considerable overlap in the physiotherapeutic actions of the intervention and control group. Process evaluation based on the quantitative video information revealed that the time during intervention spent on coaching of the family and on varied motor activities during which the infants' capacities were challenged, were associated with better motor development and functional mobility at 18 months CA (113, 114). Currently, a replication randomized controlled trial on the effect of COPCA on developmental outcome and family function in infants at very high risk for CP is carried out (118). Overall, the reviewed studies indicate that virtually no evidence is available on the effect of early intervention in infants developing CP, as the studies available were underpowered or suffered from overlap in the contents of intervention in study and control group (119, 120).

Other studies evaluated the effect of intervention in children with CP or with a high suspicion of CP, starting the intervention at about 1 year of age (range 4–36 months CA). The oldest study was performed in the seventies of last century (121). It evaluated in 24 infants whom mostly developed dyskinetic CP, whether neurophysiologically based physical therapy, including parent training and applied twice a month, affected the child's motor development more than equally frequently applied therapy consisting of passive movements to promote joint mobility. The intervention started between 5 and 17 months (median: 11 months) and outcome was assessed at the age of 2 years. The results showed that outcome in both group was similar; however, also this study was underpowered. Mayo (122) evaluated in the eighties of last century in 29 infants who mostly developed spastic CP, whether 6 months of NDT provided once a week had a better effect on a non-validated, complex aggregated parameter of motor outcome than 6 months of NDT provided once a month. The infants entered the study between 4 and 18 months of age (median 12: months). The study indicated that the higher dosage of NDT (once a week) was associated with better motor development. The Palmer et al. study (123) was also carried out in the eighties of last

century. This randomized controlled trial included 48 children aged 12–19 months with bilateral spastic CP (diplegic type). The study compared the effect of 1 year of NDT provided in two sessions per month with a home-based infant-stimulation program that included motor, sensory, language, and cognitive activities of increasing complexity. At the end of the intervention, motor development was significantly better in the children who had received the infant-stimulation program than in those who had received NDT. Cognitive development in both groups was similar. This well designed study with little attrition provided good evidence that an infant-stimulation program is associated with better motor outcome than NDT. Finally, Reddihough et al. (124) evaluated in the nineties of last century in a group of 66 children with CP, whether 6 months of conductive education provided for 8 h/week had a more beneficial effect on motor development than traditional therapy provided with a similar intensity. The children aged 12–36 months at study entry (mean: 22 months). Outcome measured with the Gross Motor Function Measure at the end of intervention was similar in both groups. Only part of the study was carried out with a randomized assignment to intervention or control group, which reduced the study's validity. Overall, only one of the four studies, which evaluated the effect of early intervention that started after the age of 3 months CA provided evidence on the effect of early intervention; the Palmer et al. study (123) provided moderately strong evidence that intervention by means of an infant-stimulation program provided twice per month was associated with better motor outcome than equally frequent intervention by means of NDT.

Recently, a large randomized controlled trial ($n = 128$) compared the effect of 6 months of context-focused therapy with that of a similar period of child-focused therapy in preschool aged children with CP [mean age at study entry: 3.5 years (125)]. After the intervention, gross motor function and daily life function had improved in both groups, but in both groups to a similar extent. The finding of a similar outcome in this high-quality study is intriguing, as the theoretical background of both approaches is substantially different – in the context-focused therapy the family and functional performance are key notions in the intervention, whereas the child-focused approach emphases improvement of the child's movement skills. The outcome of the study stresses the need of detailed process evaluation in early intervention studies, and the need of interventions that are tailored to the specific impairments of the child and the specific needs and wishes of the family. Goal directed functional therapy may offer one of the means to improve the success of early intervention (126, 127).

Next to the more general programs of early intervention, also specific interventions have been developed. For instance, Mattern-Baxter et al. (128) recently studied the effect of 6 weeks of intensive home-based treadmill training in 12 young children with CP (aged 9–36 months, mean: 21 months; Gross Motor Function Classification System levels I and II). The intervention group of six infants received twice daily during 6 days/week treadmill training sessions of 10–20 min. During the intervention period of 6 weeks, the infants continued to receive their usual therapy. The six control infants received only their usual therapy. Direct after the intervention, but also 4 months post-intervention function in daily life of the infants who had received treadmill training was significantly better than that of the control group. Currently, other specific approaches of early intervention are studied. The upper limb baby early action-observation training (UP-BEAT) study is an example (129). It studies the effect of action–observation training in infants with an asymmetric brain lesion, as action–observation has been shown to promote bimanual function in older children with unilateral CP (130). Action observation is based on the idea that new motor skills can be learned by observing motor actions, a coupling, which is facilitated by the function of the mirror neuron system (131). Another promising approach is the application of constraint-induced movement therapy (CIMT) in infancy [Baby-CIMT (132)]. CIMT is known to be effective in promoting bimanual activities in older children with unilateral CP, an effect, which, however, also may be achieved by bimanual training (133).

CONCLUDING REMARKS

The lesions of the brain underlying CP vary substantially in size, site, and time of occurrence. Most lesions occur between 24 weeks PMA and term age, a period during which developmental activity in the brain reaches its summit. Variations in timing of the insult to the brain not only result in different lesions but also in different neuroplastic reactions and different associated neuropathologies. This turns CP into a very heterogeneous entity. This may mean that the best early diagnostics and the best intervention methods may differ for various subgroups of children with CP.

Currently, prediction of CP in early infancy is best when based on multiple assessment techniques and series of assessments. In infants admitted to a NICU, the combination of neonatal imaging of the brain and GMA results in best prediction of CP. In infants who are not admitted to neonatal intensive care services, careful documentation of milestones in combination with a neurological assessment currently is the best – but non-optimal way – to detect infants developing CP.

Knowledge on the effect of early intervention in infants with severe CP is conspicuous by its absence. Animal studies suggest that the opportunities for a beneficial effect of intervention after a severe lesion of the brain are limited (33, 34). This does not mean that hope for improvement of function is nil. But, it may mean that we should encourage families to use already at early age assistive devices. Examples are adaptive seating devices, which promote upright sitting and therewith a better orientation in the environment (134), and power mobility, such as modified ride-on toy cars, allowing for exploration of the environment (135). The assistive devices may promote the children's social and cognitive development (136). Information on the effect of the assistive devices is urgently needed.

The studies in high-risk infants without CP indicate that early intervention has a stronger effect on cognitive development than on motor outcome. This result is in line with the animal studies on early intervention (33, 34). The effect on cognitive outcome is, however, essential as cognitive function determines to a much larger extent activities and participation of children with CP than motor function (137, 138).

The early occurrence of the lesion of the brain in CP offers opportunities for early intervention. The evidence that early intervention is able to improve developmental outcome in children with CP is, however, very limited. Weak evidence suggests that

parental coaching and provision of hints and suggestions on how to challenge infant activities during daily life – characteristic elements of the COPCA program (110, 111) – are associated with improved functional outcome. Moderate evidence indicates that in infants older than 1 year infant stimulation has a better effect on motor development than NDT. On the other hand, weak evidence suggests that a higher dosage of NDT used in infants older than 1 year may result in a better outcome than a lower dosage. In older children with CP, it is well-known that high dosages of therapy have a better effect than low dosages (133). The most elegant and most efficient way to achieve high dosages of specific activities is by integrating practice into daily life activities. This underscores the need of family centered early intervention. This may not only improve body function but, more importantly, also activities and participation.

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Movement recognition technology as a method of assessing spontaneous general movements in high risk infants

Claire Marcroft [1,2] ****, Aftab Khan*** [3] ***, Nicholas D. Embleton*** [1] ***, Michael Trenell*** [2] ***and Thomas Plötz*** [3]

[1] *Neonatal Service, Royal Victoria Infirmary (RVI), Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK*
[2] *MoveLab, The Medical School, Newcastle University, Newcastle upon Tyne, UK*
[3] *Culture Lab, School of Computing Science, Newcastle University, Newcastle upon Tyne, UK*

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Catherine Disselhorst-Klug, RWTH Aachen University, Germany
Mijna Hadders-Algra, University Medical Center Groningen, Netherlands

****Correspondence:***
Claire Marcroft, Newcastle Neonatal Service, Special Care Baby Unit (Ward 35), Royal Victoria Infirmary (RVI), Newcastle upon Tyne NE1 4LP, UK
e-mail: c.marcroft@newcastle.ac.uk

Preterm birth is associated with increased risks of neurological and motor impairments such as cerebral palsy. The risks are highest in those born at the lowest gestations. Early identification of those most at risk is challenging meaning that a critical window of opportunity to improve outcomes through therapy-based interventions may be missed. Clinically, the assessment of spontaneous general movements is an important tool, which can be used for the prediction of movement impairments in high risk infants. Movement recognition aims to capture and analyze relevant limb movements through computerized approaches focusing on continuous, objective, and quantitative assessment. Different methods of recording and analyzing infant movements have recently been explored in high risk infants. These range from camera-based solutions to body-worn miniaturized movement sensors used to record continuous time-series data that represent the dynamics of limb movements. Various machine learning methods have been developed and applied to the analysis of the recorded movement data. This analysis has focused on the detection and classification of atypical spontaneous general movements. This article aims to identify recent translational studies using movement recognition technology as a method of assessing movement in high risk infants. The application of this technology within pediatric practice represents a growing area of inter-disciplinary collaboration, which may lead to a greater understanding of the development of the nervous system in infants at high risk of motor impairment.

Keywords: preterm birth, cerebral palsy, neuro-motor assessment, general movement assessment, movement recognition

INTRODUCTION

Each year more than 15 million babies worldwide are born preterm (before 37 weeks gestational age) and the number of cases continues to rise (1). Infants born preterm are at higher risk of developing motor impairment than infants born at term (2). Morbidity is inversely correlated to gestational age meaning that those born extremely preterm (<28 weeks gestation) are most at risk (3). Cerebral palsy (CP) is a common motor impairment (3) for high risk infants (such as those born preterm) and these infants are also at high risk of developmental delay and other motor coordination disorders (4).

There are currently no standardized clinical guidelines for the prediction of motor impairment in high risk infants and the identification of those at highest risk typically involves the integration of clinical history, neuroimaging results, different clinical assessments, and experience of health care professionals. The assessment of spontaneous general movements is an important tool, which can be used for the prediction of movement impairments in high risk infants (5).

Movement recognition aims to capture and analyze relevant limb movements through computerized approaches focusing on continuous, objective, and quantitative assessment. Different methods of recording and analyzing infant movements have recently been explored. Camera-based solutions (6–10) and body-worn miniaturized movement sensors (11–14) have been applied in order to record continuous time-series data that represent the dynamics of limb movements. Various machine learning methods have been developed to analyze the recorded movement data. This has specifically focused on the detection and classification of atypical spontaneous general movements.

The aim of this article is to briefly summarize the current most evidence based clinical approach to observational movement assessment in high risk infants (Prechtl's General Movements Assessment) and identify the current studies, which have applied a variety of automated movement recognition technologies to assess infant movement.

CLINICAL PREDICTION OF CEREBRAL PALSY USING PRECHTL'S GENERAL MOVEMENTS ASSESSMENT

Infants learn how their bodies move and interact with the environment in early infancy (15). This is achieved through the development of spontaneous movements into goal directed movements through exploration and problem solving (15). Consequently, the development of spontaneous movements in early

infancy is a high predictor for later movement (and also cognitive) performance (4, 16).

The development of spontaneous movements in infants has been studied and described in detail by Heinze Prechtl and colleagues (17). Prechtl's general movements assessment is currently the clinical assessment that can most reliably predict CP in high risk infants with a reported sensitivity of 98% (95% confidence interval, CI 74–100%) and specificity of 91% (95% CI 83–93%) (5).

The application of this assessment clinically involves the evaluation of the qualities of the spontaneous general movements through Gestalt perception of the observer (18). A video recording is taken of the infant's spontaneous general movements, which is then assessed during playback of the video recording at normal speed and in addition, a higher speed may be used to identify the presence of movement stereotypies (18).

In typically developing infants, spontaneous movements are characterized by large variation (18, 19). Writhing movements (observed between 36 weeks and 2 months post term) are performed with moderate amplitude and speed and are characterized by high complexity and large variation in relation to amplitude, velocity, and acceleration (18). Between 2 and 5 months of age, fidgety movements become apparent: these show smaller amplitudes of circular shape, lower speed, and a higher variability in acceleration (18).

Atypical motor development is characterized by limited variation and limited variability in generalized movements (18). In particular, the presence of cramped synchronized general movements (CSGMs) during preterm and term age and the absence of fidgety movements at 3–5 months are strong predictors for later CP diagnosis (20, 21). CSGM's are atypical and lack fluency, variation, and complexity and are also stereotyped in nature (limb and trunk muscles contract and relax nearly simultaneously) (18, 21).

The challenges of applying the GM assessment in practice relate to the availability of appropriately trained and skilled clinicians. Considerable training is required for an assessor to become reliable enough to make an accurate evaluation. The assessment is susceptible to observer fatigue (21) and is dependent on the behavioral state of the infant during recording (ideally an infant should be in an alert, awake state). By the time, a single GM assessment is most accurate in predicting CP (3–5 months) (5) an opportunity to influence the nervous system at an earlier stage of development may have been missed.

Despite good levels of inter-observer reliability with the GM assessment (22), there will always remain a degree of subjectivity in interpretation. In common with all techniques involving interpretation by a skilled and experienced observer (including for example, ultrasound scan interpretation), experience of interpretation will improve with time. Despite this, it is not possible to determine the nature and extent of any subjectivity; however, the lack of widespread adoption among clinical teams may suggest there is a concern.

These challenges in the early detection of motor impairments in high risk infants have led to an increasing interest in the use of automated movement recognition technologies being applied in this clinical area.

AUTOMATED MOVEMENT RECOGNITION FOR CLINICAL MOVEMENT ASSESSMENT

The use of automated movement recognition technology has been explored in many different clinical conditions such as dementia (23), Parkinson's disease (24), and Autism (25, 26). Many of these applications have produced promising results regarding the overall potential for continuous and longitudinal assessments in both clinical and home environments (27).

Movement recognition in clinical applications aims at the automated detection, classification, and assessment of the quality of limb movements focusing on indications for abnormalities. Capturing limb movements can be based on either indirect or direct sensing (28). Indirect sensing utilizes devices that are integrated into the assessment environment, such as video cameras or 3D motion capture facilities (29) and direct sensing records movement data through sensors that are worn by the patient, i.e., miniaturized devices attached to the limbs (11, 30, 31). Both methods capture movement as time-series data, i.e., sequences of sensor readings. Depending on the sensing modality applied, this data exhibit differences in temporal and spatial resolution, which requires adaptations of the analysis methods. Typical methods for automated data analysis include sequential probabilistic modeling, e.g., using Markovian approaches (32), statistical methods (11, 14) to model specific gestures of interest (12) or holistic monitoring of general movements (27).

There are considerable challenges in utilizing this technology in the neonatal environment, in particular, the size, fragility, and vulnerability of infants needs to be taken into account. Furthermore, the automated movement assessment must be sensitive and reliable enough to detect subtle changes in movements, which are the basis for clinical diagnosis.

Table 1 summarizes some of the advantages and disadvantages associated with the two sensing modalities, i.e., direct sensing, where movements are captured using hardware directly attached to the subject; and indirect sensing, where hardware is placed in the assessment environment.

Figure S1 in Supplementary Material gives an overview of the general approach to automated gesture recognition for clinical movement assessment. Sensory data for capturing movements using one or more of the sensing techniques (summarized in **Table 1**) are usually *pre-processed* [see in Ref. (40, 48, 49)] for wearable sensors and (50, 51) for vision-based systems followed by movement *segmentation*, i.e., parts from the recorded data are partitioned that might contain important movement information [see in Ref. (26) for automatic segmentation]. This is usually followed by *feature extraction* used to represent large amounts of sensing data in a reduced fashion [e.g., (52); see also Ref. (53) for a generic feature learning approach]. Finally, *classification* is performed to identify (or predict) different types of movements. Various classification frameworks have been reported in the context of wearable/environmental sensors such as (54–56) and vision-based systems for activity recognition (57–59). A supervised classification framework also requires some form of ground truth labeling. These labels are used for training a machine learning classifier [some issues related to ground truth annotation have been addressed in Ref. (48, 60); automated vision-based annotation systems have also been explored in Ref. (59, 61)].

Table 1 | Brief overview of some of the advantages and disadvantages associated with various sensing modalities in the context of recording general movements in preterm infants.

		Advantages	Disadvantages
Indirect sensing	Video cameras (33, 36, 63)	1. Easy to understand 2. High spatial resolution 3. High context information 4. Portable 5. High availability	1. Computationally expensive analysis 2. Privacy concerns 3. Large disk space requirements 4. Generally low temporal resolution 5. Occlusion issues
	3D motion capture (29, 37)	1. High spatial resolution 2. Depth information 3. Accurate motion capture 4. High reliability 5. High temporal resolution possible 6. Secondary movement analysis possible (such as force and weight exchange)	1. High costs 2. Computationally very expensive analysis 3. Privacy concerns 4. Very large disk space requirements 5. Large physical space requirement 6. Markers needed for motion capture 7. Occlusion issues
	Microsoft kinect (35, 47, 62)	1. High spatial resolution 2. Depth information 3. Low-cost 4. Marker-less motion capture	1. Not suitable for infants (<4 years) 2. Occlusion issues 3. Low temporal resolution 4. Limited field of view
Direct sensing	Wearable movement sensors (11–13, 34, 39–43, 45, 64)	1. High temporal resolution 2. Low-cost 3. Energy efficient 4. Privacy preserving 5. Small physical size 6. Good battery life (embedded) 7. High availability (e.g., mobile phones) 8. Actigraphs: sleep/wake patterns	1. Low spatial resolution 2. Occasional data losses (wireless) 3. Limited battery life (wireless/real-time) 4. Difficulty in consistent positioning 5. Comfort issues 6. Relative movement capture only
	Magnet tracking system (31, 46, 66)	1. High temporal resolution 2. Very high accuracy 3. Metal tolerant 4. No line of sight occlusions	1. High costs compared with accelerometers 2. Computationally very expensive analysis 3. Complex setup 4. Magnetic and electrical interference issues

In the following sections, we identify and classify existing systems for gesture recognition based automated movement assessment in preterm infants.

AUTOMATED MOVEMENT RECOGNITION FOR CLINICAL MOVEMENT ASSESSMENT IN HIGH RISK INFANTS

VIDEO-BASED ASSESSMENT

Existing video-based movement assessment systems for infants can be categorized into: (i) using three dimensional (3D) motion capture systems; and (ii) using traditional color cameras. Motion capture based systems require special markers to be attached to the limbs being tracked. High-end cameras typically provide very high 3D tracking accuracy and resolution (both spatially and temporally; Figure S1 in Supplementary Material), but at a considerable price and setup effort. These systems are most commonly seen in the research setting and due to practical limitations are not easily adaptable to the clinical environment.

Meinecke and colleagues applied a motion capture system to objectively measure the spontaneous movements of infants during the first months of life (29). Fifty-three movement-based parameters were automatically extracted from motion tracks followed by cluster analysis based on Euclidian distances that selected eight of the parameters. These were able to delineate between healthy and at risk infants. Classification was performed using quadratic discriminant analysis (sensitivity 1, specificity 0.7) (see **Table 2**). With a similar setup Kanemaru and co-workers analyzed spontaneous movements in infants aiming to investigate the relationship between spontaneous movements and the development of CP at 3 years of age. The authors found that the jerkiness in spontaneous movements at term age (defined as the time integral of the square of the magnitude of jerks per unit movement distance) was higher in infants who developed CP (8–10).

Despite the popularity of consumer 3D cameras such as Microsoft's Kinect (47), these have not yet been used extensively for movement analysis in infants. This is in stark contrast to other clinical applications (35, 62). Although having great potential for general movement analysis, Kinect's capabilities are not largely explored with regards to assessments of preterm infants. This could be because the provided human tracking system in Kinect, necessary for detailed analysis, is recommended for tracking humans who are at least 4 years old and therefore considered unsuitable for use with infants.

Table 2 | Identification of different automated gesture recognition systems applied to objectively measure movement in infants.

Maintainer	Movements/ predictions	Clinical outcome	Sensing technology	Data analysis		Dataset/ study	Results/findings[a] (sensitivity, specificity)	Reference
				Data preprocessing	Classification method			
RWTH Aachen, Germany	GM/ 1. Healthy 2. At risk	24 months (CP/no CP)	Accelerometry 19 Healthy 4 High risk	32 features using velocity + acceleration (73, 74)	Decision trees	23 Infants	(1.00, 0.86)	(45)
Seirei Christopher University, Japan	SM/ 1. With BI 2. Without BI	Nil doc	Accelerometry 7 High risk 7 Low risk	MEM + FNN + MLE + Amplitude adjusted Fourier Transform	Mann–Whitney *U test* + *Student's t-test*	14 Infants	With BI – high dimensional, unstable, and unpredictable movement	(13)
UC Irvine, USA	GM/ 1. CSGM present 2. CSGM absent	Nil doc	Accelerometry	Statistical features using acceleration including; mean, standard deviation, min, max, products, *z-value*	DT + SVM + DBN-RF	10 Infants 6 CSGM present 4 CSGM absent	(0.103, 0.939) + (0.069, 0.964) + (0.498, 0.764	(11)
University Children's Hospital Bern, Switzerland	SM/ 1. Only healthy	Nil doc	Accelerometry	Detrended fluctuation analysis (DFA)	*t-test statistical test* + *Linear regression* + *generalized least squares regression*	22 Healthy infants	Correlation study	(14)
UC Irvine, USA	GM/ 1. CSGM present 2. CSGM absent	Nil doc	Accelerometry	Several basic motion features using acceleration + mean, max, min, SD, *z-score*	RF + Boosted NB + SVM + EC/DBN	10 Infants	(0.72, 0.57)	(12)
NTNU, Trondheim, Norway	GM/(Visualization)	24 months CP/no CP	Accelerometry + Computer vision	Periodicity + PCA	n/a	14 Patients 4 FM types	n/a (visualization only)	(69)
University of Heidelburg, Germany	GM/ 1. CP 2. Non-CP	Nil (31, 52) 24 mon (46)	Magnet tracking system + computer vision	Stereotypy score, Periodic and Torpid leg movements	*t-test*	67 Infants 49 High risk 18 Low risk	(0.90, 0.95)	(31, 46, 66)
NTNU, Trondheim, Norway	GM/ 1. Healthy 2. At risk	Not specified	Accelerometry + Computer vision	Skewness, cross-correlation, areas calculated using the moving average, Periodicity, PCA, AR + Linear Separability (scatter matrix), and Clustering analysis	1. LDA 2. QDA	81 Infants	(0.86, 0.90)	(70)

(Continued)

Table 2 | Continued

Maintainer	Movements/ predictions	Clinical outcome	Sensing technology	Data analysis		Dataset/ study	Results/findings[a] (sensitivity, specificity)	Reference
				Data preprocessing	Classification method			
University of Tokyo, Japan	SM/ 1. CP 2. Non-CP	3 years Dev Delay	3D Motion capture	6 Movement indices (using Frame-DIAS; DHK, Japan) including Jerk index (time integral of the square of the magnitude of jerks per unit movement distance)	Kruskal–Wallis test + Fisher's exact test + Mann–Whitney *U test*	145 Infants 16 CP 129 Normal	Significantly higher jerk index in CP	(8–10)
St. Olav University Hospital, Trondheim, Norway	GM/ 1. CP 2. Non-CP	5 years (CP/no CP)	Computer Vision	Quantity of motion, Centroid of motion, Variability of velocity and acceleration, CP predictor feature	*t-test statistical test + Mann–Whitney U test + Logistic regression*	30 High risk infants	(0.85, 0.88)	(7)
St. Olav University Hospital, Trondheim, Norway	GM/ 1. FM present 2. FM absent	Nil doc	Computer Vision	Quantity of motion, Centroid of motion, Variability of velocity and acceleration	Threshold analysis	82 Infants 50 Low risk 32 High risk	(0.815, 0.70)	(6)
RWTH Aachen, Germany	GM/ 1. Healthy 2. At risk	Nil doc	3D Motion capture	Skewness, cross-correlation, area outside the SD of moving average, Area differing from moving average + Cluster analysis with Euclidian distances	QDA	22 Infants 15 Healthy 7 Affected	(1.00, 0.70)	(29)

GM, general movements; SM, spontaneous movements; CSGM, cramped synchronized GM; CP, cerebral palsy; BI, brain injury; PCA, principal component analysis; SD, standard deviation; DT, decision tree; NB, naïve Bayes; MEM, maximum entropy method; FNN, false nearest neighbors; MLE, maximal Lyapunov exponent; AR, auto-regression; SVM, support vector machine; QDA, quadratic discriminant analysis; LDA, linear discriminant analysis; EC/DBN, Erlang-Cox/dynamic Bayesian network.

[a] *Predictive values of the assessment methods listed in the table need to be interpreted with caution as the number of infants included in the studies are mostly low.*

Standard video cameras such as regular web-cams, RGB cameras on tripods, and even video-enabled baby monitors (36) have also been used for marker-less capturing of infants' body movements. These systems offer a more reasonable priced alternative to 3D motion capture system and in addition come with substantially less set up effort, which enables applications beyond research and clinical settings, allowing for continuous and more detailed analysis in natural (home) environments. Typically, these recording setups come with lower spatial and temporal resolution, which limits the level of detail of the analysis. Additionally, marker-less tracking is less accurate than professional motion capture (37), which may be problematic when attempting to identify more subtle differences in movement patterns. An example would be in identification of the differences between CSGMs and poor repertoire general movements. CSGMs are described as monotonous and rigid with the limb and trunk muscles contracting and relaxing simultaneously (33) and poor repertoire general movements are monotonous, with the amplitude, speed, and intensity lacking normal variability (33). While both of these movement patterns are identified as being atypical (18, 33), CSGMs can also be most easily recognized by human gestalt perception and also have a much higher predictive value for motor impairment (33). Therefore, identification of any difference between the two atypical patterns is clinically highly relevant.

Adde and colleagues have developed an advanced video-based analysis system for quantitative and qualitative assessments of infants general movements (63). Utilizing standard RGB video cameras and an analysis method that uses so-called "motion-grams" for quantifying changes in the infant's movements, the general movements toolbox (GMT) is able to detect fidgety movements as described in the GM assessment (18). This clinical toolbox may be useful in the early diagnosis and/or risk stratification of infants at high risk of developing CP. The first studies using GMT on a small number of high risk infants ($n = 30$) report promising results (sensitivity 85% and specificity 88%) with respectable follow-up data past 4 years of age (7). This suggests that the application of the GMT as a method for prediction of neurological impairment may be straightforward, cost-effective, and feasible for use in clinical practice but will require further systematic validation.

ASSESSMENT THROUGH DIRECT MOVEMENT SENSING

While indirect sensing settings require external tracking equipment, body-worn accelerometers have recently been successfully applied and remain popular in clinical studies. In particular, the proliferation of affordable, reliable, and miniaturized sensing facilities in combination with sophisticated data analysis techniques has allowed for automation of movement assessment in small infants. Accelerometers are sensors, which measure inertial forces in one or three spatial axes, resulting in high-resolution time-series data that represent the dynamics of the acceleration of the sensor during movements (34). Specifically, they have been used to measure physical activity levels in children (34), sleep/wake cycles, and the physiology of swallowing in infants (38, 64). These applications are in line with a large body of research that focuses on objective assessments of movement related parameters in a multitude of health-related scenarios, including gait analysis (39) sport activity (40), rehabilitation monitoring (41, 42), quantification of disease progression, and investigation of effectiveness of therapy interventions (43).

Even though reduced frequency and quantity of limb movements in infants have recently been identified as early predictors for developmental delay (9), it is widely accepted that in infants with a suspected diagnosis of CP, the general movements change in quality rather than quantity (44, 65). An automated analysis therefore needs to capture fine details of limb movements, for which video-based assessments are often limited.

Karch et al. developed an electromagnetic tracking system to undertake movement analysis (66). Small lightweight sensors (1.3 mm diameter) were attached to the infant's limbs, which were then sensed using a commercial (external) tracking system that provided high accuracy and allowed for detailed analysis of joint flexion. Serving as proof of concept this tracking system has successfully been used for detecting anomalous, spontaneous limb movements in infants (31, 66). Phillippi et al. conducted a recent study using this work where it was found that the stereotypy score of arm movements could be used as a predictor for CP and stereotyped periodic leg movements predicted neurodevelopmental impairments (46).

A limited number of more recent studies have successfully applied accelerometers to preterm infants to measure spontaneous movements (13, 31, 45, 46) and also to create models for atypical movement patterns such as CSGMs (11, 12). While this is an evolving area of translational use of technology in the healthcare setting, there is limited data quantifying limb movements and also comparing the results to longer-term neurodevelopmental outcomes. Ohgi et al. used tri-axial accelerometry for a cross-sectional study that measured upper limb acceleration in a small number of preterm infants with and without brain injuries at 1 month corrected age (13). This was the first application of accelerometers in preterm infants in a clinical environment but the recording time was limited to 200 s due to the variability of the infant's states. Statistical analysis confirmed that infants with brain injuries exhibited unstable and unpredictable spontaneous movements with larger dimensionality (details of the analysis techniques and statistical tests are provided in **Table 2**).

A group from University of California, Irvine have tested the use of wireless accelerometers in the neonatal unit and compared accelerometer data to the general movements assessment (11, 12). CSGMs are highly predictive of CP (21, 67) and the group investigated whether accelerometry, combined with machine learning techniques for automated data analysis, could accurately identify the components of these atypical movements. By means of this automated assessment, limb acceleration and correlation have been characterized successfully. Gravem et al. used various statistical classification techniques to correctly identify the presence of CSGM in 6 out of 10 infants (11). Reported accuracy varied between 70 and 90% (see **Table 2** for details).

Heinze et al. used miniature accelerometers with the aim of developing a methodology to allow objective diagnosis of the development of movement disorders in preterm infants (45). The overall detection rate was >90% over three measurements and the timing of the data collection correlated with the characteristics of spontaneous movements as described in the general movements

assessment (18). Nineteen healthy term infants and four "at risk" preterm infants were included in the study. Clinical and neurological examinations were undertaken and "at risk" infants were identified through abnormalities on computer tomography and/or follow-up to 2 years.

These direct sensing studies represent promising applications of technology to contribute to the identification of atypical movement patterns in preterm infants. However, it is important to take into consideration that only small numbers of preterm infants with atypical movements were included which do not allow for the calculation of sensitivity and specificity. Furthermore, the feasibility of applying these techniques to larger numbers of high risk infants in a clinical environment is difficult to establish and in comparison to applying video-based methods of assessment (including Prechtl's GM assessment) may be more difficult to achieve due to the logistics of attaching direct sensors to small infants.

Hybrid systems

The main advantage of video-based movement analysis lies in the recording of spatial data in addition to temporal measurements, which gives direct access to, for example, posture-related information. In contrast, direct sensing approaches typically have a much higher temporal resolution allowing for more detailed assessments, which is beneficial for in-depth analysis of subtle changes that may precede the development of CP.

Some approaches have been developed that aim at combining both sensing methods in order to benefit from both spatial and high temporal resolution – at reasonable costs and minimum setup efforts for practical applications – for high-fidelity movement analysis (68). Berge and colleagues have proposed a software tool for GM representation and modeling called ENIGMA – enhanced interactive general movement assessment. ENIGMA provides a useful support tool for visualizing features of motion data in conjunction with video data for GM experts (69). Similarly, Rahmanpour et al. employ a hybrid sensing approach for the prediction of CP using an extensive analysis of movement-based features. The authors found that dynamic features are more indicative than the standard statistical features (70). Results from their analysis are shown in **Table 2** with a detailed description of the employed features.

SUMMARY

Prediction of motor impairment (such as CP) in preterm infants is challenging, and ideally requires techniques that are both sensitive and specific. Due to the large number of complex factors affecting neurodevelopment, and the difficulty in assessing brain plasticity, predicting which children will develop CP on the basis of a single assessment will always be challenging (71). A multimodal longitudinal approach including a combination of methods, e.g., neurological assessment, general movements' assessment, and neuroimaging is likely to improve both positive and negative prediction. Early risk stratification and prediction has many benefits. It allows for early identification of those most likely to benefit from early intervention and targeting of resources and support for parents. Furthermore, access to health, social, and educational services is often dependent on a diagnosis (72).

Accurate, non-invasive assessments of sufficient sensitivity to identify longitudinal changes in movement patterns could hold considerable hope for the future. This mini-review has identified recent studies employing video-based assessment, assessment through direct movement sensing and hybrid systems. Specifically, the use of accelerometry and computer vision may offer clinically feasible and promising methods of objectively measuring quality and quantity of infant movement. The application of these technologies may prove to be useful not only in the prediction of infants at highest risk of motor impairment but also in the evaluation of therapies aiming to influence the developing brain.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fneur.2014.00284/abstract

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Technology-aided assessment of sensorimotor function in early infancy

Alessandro G. Allievi[1†], Tomoki Arichi[1,2†], Anne L. Gordon[3,4] and Etienne Burdet[1*]

[1] Human Robotics Group, Department of Bioengineering, Imperial College London, London, UK
[2] Department of Perinatal Imaging and Health, King's College London, London, UK
[3] Paediatric Neurosciences, Evelina London Children's Hospital, Guy's and St Thomas' NHS Trust, London, UK
[4] Institute of Psychiatry, Psychology and Neuroscience, Kings College London, London, UK

Edited by:
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***Correspondence:**
Etienne Burdet, Department of Bioengineering, Imperial College London, South Kensington Campus, London SW7 2AZ, UK
e-mail: e.burdet@imperial.ac.uk

[†]Alessandro G. Allievi and Tomoki Arichi have contributed equally to this work.

There is a pressing need for new techniques capable of providing accurate information about sensorimotor function during the first 2 years of childhood. Here, we review current clinical methods and challenges for assessing motor function in early infancy, and discuss the potential benefits of applying technology-assisted methods. We also describe how the use of these tools with neuroimaging, and in particular functional magnetic resonance imaging (fMRI), can shed new light on the intra-cerebral processes underlying neurodevelopmental impairment. This knowledge is of particular relevance in the early infant brain, which has an increased capacity for compensatory neural plasticity. Such tools could bring a wealth of knowledge about the underlying pathophysiological processes of diseases such as cerebral palsy; act as biomarkers to monitor the effects of possible therapeutic interventions; and provide clinicians with much needed early diagnostic information.

Keywords: cerebral palsy, motor assessment, developmental assessment, robotic-assisted assessment, MRI, functional MRI

The first 2 years of human childhood are a crucial period for the establishment of the key neural circuits, which subserve the development of normal motor function in humans. Although the corticospinal tracts project into the spinal cord relatively early during *in utero* life (approximately 24 gestational weeks), the final pattern of life-long connectivity is established during the first few post-natal years, through activity-dependent mechanisms, which influence the critical balance between the projection and withdrawal of axons (1). An injury to the developing brain at this critical juncture can result in cerebral palsy, which collectively describes the resulting motor disorder consisting of impairments of posture and movement control (2). While the pathophysiology of motor dysfunction is complex and multi-factorial, recent advances in non-invasive imaging now allow the cause of the majority of cases to be identified as perinatally acquired brain lesions such as those seen following perinatal asphyxia, hemorrhagic or ischemic stroke, and preterm birth (3). Although the resultant motor impairments can be managed (and in some cases improved) with a variety of targeted therapies, the condition remains non-curable and most importantly, there appear to be no bio-physiological mechanisms in place for spontaneous healing or recovery. This apparent failure is perhaps surprising given that neural plasticity is generally considered to be at its most mouldable state during early infancy, suggesting that there should be an increased potential at this stage of life to compensate for neural damage via the retention/formation of alternative axonal routes and synaptic connections (4–11). Early diagnosis of cerebral palsy thus becomes fundamental as it would allow the early identification of appropriate candidates for interventional strategies and also ensure a more efficient allocation of long-term healthcare resources and social support.

In this review, we will describe how novel technology-assisted assessment solutions have the potential to integrate and expand on currently available clinical assessment tools of sensorimotor function, and may provide a sensitive and accurate means of diagnosing cerebral palsy within the first 18 months of post-natal life. Furthermore, we will discuss how these can provide vital new information about the underlying pathophysiology of the disease; thereby also improving our understanding about the mechanisms of and the factors which influence neural recovery (and potential plasticity) following brain injury. The quantitative information potentially provided can also be used to directly monitor the effects of therapeutic intervention both in clinical practice and in the research setting, and in the case of imaging, visualize their effects. To put the techniques in context, we first briefly review currently used clinical assessment tools, and then the small body of studies, which describe using "technology-assisted" tools to obtain objective measurements of how very young children interact with toys fitted with movement sensors. We consider the predictive utility of Magnetic Resonance Imaging (MRI) assessment and discuss how novel task-based functional magnetic resonance imaging (fMRI) paradigms using automated robotic devices can additionally assess the functional state of the neonatal brain. Finally, despite the increasing use of such tools in adult post-stroke assessment and fMRI experiments, their use with children has been limited. We will therefore also discuss the relative benefits and draw-backs

to these approaches, and the challenges inherent to developing technology-assisted devices so that they are suitable for the young infant population.

EARLY INFANT MOTOR DEVELOPMENT AND ASSESSMENT

Within the first few post-natal months, infants rapidly acquire new patterns of posture, muscle tone, and motor behavior, with spontaneous but seemingly non-goal-orientated movements replaced by an increasing repertoire of purposeful goal-directed movements (12). This change allows the developing infant to interact in an increasingly active manner with its surrounding environment: thus allowing exploration, early learning, communication, as well as maintaining musculoskeletal integrity (13). The ongoing ontogeny of particular motor skills during early infancy is sufficiently systematic that milestones representative of their age of attainment (such as standing and walking independently) can be generally used as relatively robust markers of gross motor development. The first clinical suggestion of developing cerebral palsy is therefore often a delay in milestone attainment, or an observable deviation from typical motor behavior (such as asymmetrical hand use), both of which may not be evident until well after 12 months of age. Making a diagnosis of cerebral palsy earlier in infancy is complicated not only by the rapidly evolving and dynamic nature of early human development, but also by the relatively restricted repertoire of abilities in infants in terms of motor, cognitive, social, and communication responses on testing. Of crucial importance, however, specifically developed clinical assessment tools have found that subtle patterns of neurological abnormality can be identified from a very early age, thus highlighting the feasibility and validity of early diagnosis.

CURRENT CLINICAL ASSESSMENT TOOLS

Clinical assessment tools typically involve a single or multiple assessors (usually health care professionals who have been specially trained to administer the test) observing or interacting with an infant, and subsequently scoring the motor behavior or performance in particular tests. Scoring is generally done using ordinal scales, which may be dichotomized (i.e., yes/no reflecting an infant's ability to perform a task), or have a range of values, which reflect performance with reference to a pre-defined rank order (relative to a population appropriate spectrum of performance). Recent systematic reviews have identified neurobehavioral and neuromotor assessments suitable for use in infants and evaluated their validity and reliability at: (i) discriminating between individuals who are/are not affected by neurological or motor dysfunction, functional limitations, or disabilities at the time of assessment (discriminative ability), (ii) predicting future neuromotor performance, condition, or outcome based upon performance at the time of assessment (predictive ability), and (iii) evaluating longitudinal changes in neuromotor performance, and the impact of intervention (evaluative ability) (14, 15).

Due to the current lack of a criterion standard for neonatal assessment, all available tools are criterion referenced, and no individual tool offers the best possible discriminative, (long and short term) predictive, and evaluative properties (14). While it is proposed that combining assessments that measure different constructs may yield the best psychometric results, this approach is often impractical. A suitable tool should thus be chosen by considering the primary purpose of the assessment (14). In general, due to the statistical effects posed by the numerous environmental and developmental confounds, evaluative validity of current assessment tools has been sparsely and poorly reported (16). Of the identified assessment tools, the Test for Infant Motor Performance (TIMP) was found to be a good all-round tool (and the only suitable evaluative assessment tool), and the assessment of General Movements (GMs) to be the best long-term predictive assessment tool for the given age range (14).

The TIMP was specifically designed for the assessment of infants between 34 weeks post-menstrual age (PMA) and 4 months post-term, and consists of two scales for rating both spontaneous motor behavior and motor responses to stimulation (17). Spontaneous motor behavior is scored by 28 observed items consisting of movements such as head centering, reaching, and finger movements, while the elicited motor behavior is scored by performance in 31 items, which assess the infants response to placement, handling, and visual or auditory stimulation (17). Of particular importance, specific items in the TIMP have been formally assessed for their utility in the prediction of cerebral palsy, and have been found to correlate well with developmental outcome at 6 months of age as assessed by the Bayley Scales of Infant Development (18, 19). However, while the TIMP has been found to have excellent sensitivity (with over 90% of infants correctly predicted to develop cerebral palsy) and good specificity (with 76% of infants correctly predicted not to develop later cerebral palsy) for the prediction of motor outcome at 12 months of age, approximately 35 min are required for an experienced practitioner to administer the test, and its predictive validity has been found to differ depending on the age of testing (20, 21).

It has been suggested that the assessment of GMs may provide the most objective measurement of an infant's clinical status as it performed using a video recording of their spontaneous movements; thereby eliminating the need for patient handling and minimizing inter-rater variability (22, 23). The fundamental premise of GM assessment is that the quality and quantity of self-generated motor behavior is an accurate representation of the condition of the developing nervous fetal or infant nervous system (22). GM assessment within the first 4 months of life has been found to predict cerebral palsy at 2 years of age with an excellent sensitivity (>90%), and a good, but variable specificity (between 60 and 100%).

Given the previously discussed implications of activity-dependent processes on the nervous system development, a patient group of particular interest is young infants at high risk of developing unilateral cerebral palsy, in whom an accurate measure of early bimanual function may enable the development of interventions using the apparent window of enhanced neuroplasticity in this period. Taking this into account, the mini-Assisting Hand Assessment (AHA) tool for infants between 8 and 18 months of age at high risk of unilateral cerebral palsy is currently under development (24, 25). The aim of the mini-AHA is to transpose the established AHA clinical assessment for older children to this younger population, whilst maintaining the tool's capacity to accurately assess a child's ability to use each of their hands independently, and additionally to incorporate more cognitively demanding (and

perhaps more discriminative) bimanual tasks (25). Much like the AHA, the mini-AHA proposes to assess, discriminate, and evaluate longitudinal changes in the usage and performance of the affected hand in children with unilateral cerebral palsy. This is done by observing the children play with a series of specifically designed toys, and scoring their ability (on a four point scale) to perform twenty increasingly difficult object-related manipulative tasks, ranging from simple holding of an item to bimanual toy interaction (24). A preliminary internal-scale validation study has found that mini-AHA items can be ordered hierarchically using a Rasch model fit, such that a discrete monotonic increase is seen in both the item difficulty coefficient and the infant's ability to use their affected hand (24). Children were very finely separated according to their level of ability, and, crucially, test scores were found not to be affected by age.

The mini-AHA and in general all observation-based clinical assessment tools however, suffer from several limitations. Firstly, longitudinal studies on large population cohorts are required to validate the discriminative and predictive power of the tool. Before such tools can be made common place in clinical practice, all of the administering health care professionals must undergo extensive (and expensive) training sessions to minimize the effects that subjectivity and resultant inter-rater variability may have on the final score. As the majority of clinical assessment tools are scored using categorical or ordinal scales, they all suffer from limited resolution, affecting their ability to provide new information about the disease mechanisms and natural history. Finally, the assessment procedures are often extremely time-consuming, resulting in prolonged and costly commitments of clinical staff, and facilities.

SENSOR-BASED, AUTOMATIC ASSESSMENT TOOLS

Here, we define a "technology-assisted assessment tool" as an object or device, which has been specifically designed to automatically induce and/or precisely measure movement. In this context, an "intelligent" or "instrumented" device has been fitted with highly accurate sensors for measuring detailed aspects of motor behavior. A "robot" is equipped, in addition to sensing components, with motors for inducing controlled patterns of movement. An obvious benefit of such tools is that they provide quantitative measures of complex facets of motor function, which can then be used for both intra-subject (longitudinal) and inter-subject (both cross-sectional and longitudinal) comparison (26, 27). This could potentially allow statically robust hypothesis testing for clinical trials of novel therapeutic strategies, thereby greatly improving study power, and reducing the number of subjects required to identify a significant effect (27). Further significant advantages include removing the subjective element from any assessment, and that they can be designed to be both simple to use and time efficient, thus saving valuable clinical time and training (26).

Rather than scoring motor behavior to a relatively inflexible ordinal scale of values, the data type provided by technology-assisted devices is usually on a continuous ratio scale, thereby offering far greater resolution and potentially an important means with which to gain new insights about pathophysiology (27). Furthermore, while traditional clinical assessment provides information about aspects of motor function and behavior which can only be directly observed and felt by an examiner, technology-assisted tools can provide detailed measurements of multiple facets of motor function including those which cannot be perceived through a human observer. These include kinematic information (about the temporal and spatial quality of movement), kinetic information (about the force and work associated with motor function), and neuromechanical information (about musculoskeletal dynamics and feedback via information about impedance and viscoelastic properties) (27). Light-weight sensors and robot-assisted assessment tasks can also be designed around everyday objects and motor behavior (such as turning a door handle or pouring a drink). The derived data can then be used to calculate physiologically meaningful measures of motor behavior such as the active range of motion, target error, movement smoothness, movement time, movement deviation, and force direction error [for review see Ref. (27)].

While there is increasing evidence that robot-assisted assessment tools can complement or in some cases replace standard clinical tools in adult patients (e.g., stroke survivors), there have been very few studies which have implemented the approach to evaluate motor function in early infancy. Designing such a tool is clearly challenging as it must be non-threatening to the infant, interesting enough to encourage meaningful interaction, unobtrusive (and light-weight) enough to allow natural motor behavior but strong enough to withstand potentially rough play (**Figure 1**) (28). With this in mind, another direction consists of instrumenting toys with sensors, enabling assessment as well as a game-like interface, similar to rehabilitation robots (though without the capability to move a child's limbs). Early studies summarized in **Table 1** have created such "intelligent toys" from familiar toys equipped with light-weight sensors (28–31), that encourage play through goal-orientated activity with regular positive feedback (29, 32–35). In general, these studies have demonstrated only the feasibility of intelligent toys to derive quantitative movement measures in healthy young infants. An instrumented sorting block toy was shown to be sufficiently sensitive to demonstrate differences in performance related to motor planning and task difficulty (31). This concept has also been extended further for babies by

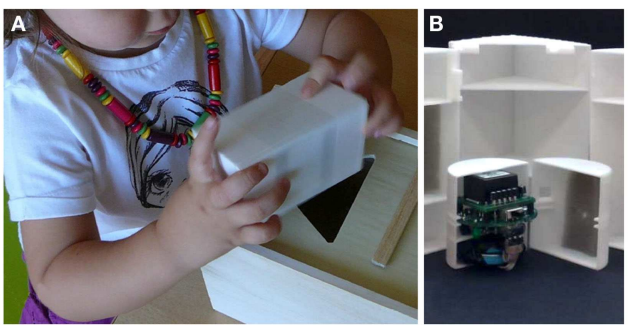

FIGURE 1 | Instrumented sorting block toy. The traditional sorting block is a relatively complex toy for young children, which ultimately requires good spatial orientation, grasp control, force control, and motion planning. In this instrumented version of the toy, the block has been equipped with an inertial measuring unit **(B)** to track and record the object's orientation and linear accelerations during grasping, manipulation, and reaching tasks **(A)**.

Table 1 | Summary of studies that have developed instrumented toys to quantitatively assess movement in young children.

Study	Design	Age group	Measures	Findings
Campolo et al. 2008 (28)	Instrumented ball toy sensorized with inertial units (accelerometer, magnetometer, and gyroscope) and custom-made force sensors (0–20 N)	6 months and above, intended for children suffering from autistic spectrum disorders	Applied force Spatial orientation and acceleration of object movement	Not formally tested with infant subjects
Cecchi et al. 2008 (29)	Instrumented rattle, sensorized with inertial units (accelerometer, magnetometer, and gyroscope) and binary contact sensors	9 months and above	Grip shape Spatial orientation and acceleration of movements	Preliminary test with three infants (24 months old) showed typical 3–4 finger grasp patterns
Cecchi et al. 2010 (34) Cecchi et al. 2010 (35) Serio et al. 2011 (32) Sgandurra et al. 2012 (36)	"Biomechatronic gym" (instrumented baby play gym) consisting of three toys (cow-toy, flower-toy, and ring-toy) integrated with visual and auditory stimuli. Toys contain piezo-resistive pressure sensors (0–5 psi) and force sensing resistors (0–20 N)	4–9 months old	Palmar (power) and precision grasp: applied pressure and force range Distinction between lateralized or centralized activity defined by position of toy during play with respect to midline	Tested longitudinally with seven infants: Central tasks: trend toward decreasing bimanual activity (and increasing unimanual activity) with increasing age for central tasks Lateral tasks: significant increase in contralateral action with increasing age Increase in occurrence of precision grasp and reduction in occurrence of power grasp with increasing age. Force applied during both grasp types increases with age
Klein et al. 2011 (30)	Instrumented block sorting toy, sensorized with force sensors, and infra-red proximity sensors	Age range not specified	Applied force on object lid as a function of shape and location Correct insertion of object, task completion time, number of mistrials, and percentage of time spent far from the target	Tested with nine blind-folded healthy adult volunteers, showed significant performance improvement with learning
Campolo et al. 2012 (31)	Instrumented block-box toy, sensorized with magneto-inertial sensors	12–36 months old	Tracking orientation during object placement Vertical and horizontal alignment errors and insertion time	Tested with four healthy infants (14–25 months old) for acceptability
Serio et al. 2012 (33)	Commercially bought horseshoe-shaped toy, sensorized with silicon chamber for pressure measurement (0–5 psi)	4–9 months old	Bimanual applied pressure during power grasp	Not formally tested with infant subjects

integrating intelligent toys into a baby gym, providing an engaging and involving enriched environment for an ecological assessment of reaching patterns and grasping forces in infants aged between 4 and 9 months (32, 34, 35). Longitudinal trials have demonstrated that this system is capable of providing quantitative measures of power grip maturation patterns in healthy infant subjects, and has the potential to be employed to objectively assess hand function in this naturally uncooperative population (34, 36). Meaningful measures about standing balance and gait in older children have also been derived from commercial gaming devices such as the Nintendo Wii, with the advantage that the device is generally familiar and attractive to the children being assessed (37).

While studies in adult stroke patients and early feasibility work in infants of technology-assisted assessment are clearly promising, there are currently no accepted standards for their implementation in clinical practice, and the majority of tools and their derived measures have not been validated or systematically evaluated (27). If the field is to be advanced, there is therefore a need to ensure that they can be optimized to collect clinically relevant information, which can be clearly related to the underlying neurophysiology. Moreover, if robot-assisted tools are to be made widely available and regularly used in clinical practice, it is also vital that their ease-of-use and cost are also carefully considered during the design.

MAGNETIC RESONANCE IMAGING ASSESSMENT

In recent years, non-invasive imaging techniques have dramatically transformed both clinical practice and neuroscience by providing a detailed means with which to visualize the causative pathology of conditions such as cerebral palsy and study the underlying disease mechanisms. Commonly used tools such as cranial ultrasound (CrUSS) can provide invaluable bed-side images of the brain during the neonatal period, thus providing clinicians with an important means with which to identify acute pathology and general anatomy. MRI scanning allows the acquisition of high spatial resolution images with excellent tissue contrast, which can be obtained in any plane (including in three dimensions), and without the risks of ionizing radiation. In addition, MRI can provide detailed visualization of the whole brain (including inferior areas such as the cerebellum, which are often poorly seen on ultrasound). This can therefore allow the precise delineation of pathology (such as hemorrhage or infarction) and its associated effects on brain structure. Moreover, the information acquired about each volume of tissue (known as a "voxel") acquired within an MRI scan is in the form of a quantifiable signal, making it highly amenable to mathematical modeling techniques and statistics. However, MRI scanning is expensive; requires for specialist staff and facilities; and the image acquisition itself is noisy and particularly susceptible to image artifacts generated by movement. Despite these draw-backs, MRI is becoming increasingly common in the clinical setting; and has established a clear place in medical and neuroscientific research due to its inherent flexibility, which allows the detailed visualization and measurement of diverse aspects of brain tissue structure, composition, and function.

FUNCTIONAL MRI AND ROBOTIC STIMULATION DEVICES

Through the measurement of temporal changes in the Blood Oxygen Level Dependent (BOLD) signal, fMRI can measure and spatially map brain activity with relatively good spatial resolution (in the millimeter range) and fair temporal resolution (usually a few seconds) (38, 39). A typical fMRI experiment consists of intermittently presenting a subject with an external stimulus (or asking them to perform a task) while a series of whole brain images are rapidly acquired; and localized areas in the brain are then identified in a subsequent (usually off-line) statistical analysis where the BOLD signal has significantly changed from baseline in a manner corresponding to the timing of the stimulus or task (40). An optimal fMRI experimental paradigm would therefore be capable of inducing a robust and repeatable change in the sampled BOLD signal in discrete regions of the brain, and at a frequency, which is clearly distinguishable from noise (40, 41). Although fMRI is now widely used in neuroscience and psychology experiments to map and characterize stimulation-induced functional activity across the whole brain, there have been relatively few systematic studies which have applied the technique to study functional activity in the developing infant brain [reviewed in Ref. (42–44)]. The majority of these studies have reported functional responses to visual and auditory stimulation (45–47), and a smaller number to tactile stimulation (48, 49). It is also of note that many of these studies used stimulus presentation methods, which were not specifically designed for infant subjects and/or were manually controlled resulting in an inconsistent pattern of stimulation.

A critical advantage of a fully automated robotic stimulus system for fMRI experiments is that it can provide a truly consistent pattern of stimulation, including precise control of the time of stimulus onset, amplitude, and frequency (50, 51). Of vital importance however, such a system must be MR-safe (i.e., poses no physical risks in all MR environments) as defined by the American Society and Material and the U.S. Food and Drug Administration (US-FDA) (http://enterprise.astm.org/) (50). In addition, all device components, which are placed inside the examination room and independently generate radio-frequency waves must be contained within their own electrically conductive shielding (known as a Faraday cage) to prevent electromagnetic interference during image acquisition (52) and the functioning of the motors should also not be disturbed by the very large magnetic field produced by the scanner. Devices should be made from non-ferromagnetic materials, and may require non-standard engineering solutions to carry out the desired action (such as using pneumatic, hydrostatic, or cable transmission) and sensing (such as fiber-optic transmission) to keep all potential interferences outside the scanner room (50), or suitable and tested shielding (53). The design of the device must also take into account the relatively small space available inside the bore of the MRI scanner, and the position of the subject (usually lying down supine). To ensure that all of the aforementioned issues are appropriately considered, the development of MRI/fMRI compatible robotic devices often requires flexible solutions and/or compromise and should involve close collaboration between engineers, MR physicists, neuroscientists, and clinicians (see **Table 2**).

We have recently developed a set of robotic devices that address these challenges, as well as a control system capable of delivering safe and reproducible patterns of stimulation for fMRI experiments with young infant subjects (43, 44, 51, 54). To ensure MRI safety, all of the components within the MR examination room are entirely metal-free, and the devices are actuated by pressurized

Table 2 | Suggested requirements for an fMRI compatible robotic device.

Contain no ferrous materials and be fully MRI/fMRI compatible
Be mechanically safe to avoid distress or possible harm to the infant
Be able to provide stimulation synchronized with fMRI acquisition
Be able to induce stimulation patterns at a controlled amplitude and frequency capable of eliciting robust functional responses
Be possible to monitor the operation of the stimulus remotely to ensure consistent stimulation was occurring and that no potentially harmful events could occur
Be light, small, and flexible enough to avoid the infant suffering movement restriction or discomfort
Not additionally induce head movements and so avoid resulting image artifacts
Be easily cleanable to prevent infection spreading from one infant to another
Be capable of presenting a stimulation type and pattern, which is appropriate for the neurodevelopmental stage of the study population

air through pneumatic tubing and a standard PC situated in the MR scanner control room (schematic of the system is shown in **Figure 2**) (43). Synchronization of the stimulation onset with image acquisition can be readily achieved by detection of the MR scanner TTL (transistor–transistor logic) pulse via the "sync" or "volume trigger" outlet port on most standard MR scanners.

Using this general system, we have been able to identify well localized and reproducible pattern of positive BOLD brain activity using a variety of stimulation paradigms and fMRI in infants during the preterm period and at term equivalent age (43, 44, 51, 54). The first of these devices was a simple latex balloon, which was placed in the palm of the infant subjects, with timed inflation and deflation resulting in opening and closing of the fingers (43). This allowed us to map brain activity in the primary somatosensory cortices of preterm infants as young as 29 weeks PMA using a block paradigm (43), and to characterize the hemodynamic response to a brief (1 s) stimulus using an event-related paradigm (54). The importance of the preterm period (equivalent to the third trimester of human gestation) was emphasized by the findings of these studies, which demonstrated systematic maturational trends in both the spatial (beginning from a predominately contralateral response in the primary somatosensory cortex in preterm infants to increasing involvement of the association motor areas with increasing age) and temporal characteristics (shorter lag time to the peak of the response with increasing age) of the identified responses (see **Figure 3**) (43, 54). These initial findings have led to significant refinement of the subject interface to allow an even finer control of the pattern of stimulation, with light-weight robots capable of providing a more specific proprioceptive stimulus with exact and highly reproducible properties to different limbs such as the wrist and ankle (see **Figure 3**) (51). Furthermore, a fiber-optic position sensor has been incorporated into the robotic interface, thus providing both precise feedback about both the pattern of stimulation and additional information about spontaneous movements made by the subject (51). We were also able to demonstrate the flexibility of the control system by adapting it to allow the presentation of olfactory stimuli, with the odor of formula milk found to induce functional brain activity in the primary olfactory areas of infants at term equivalent age (44).

STUDIES COMBINING CLINICAL AND TECHNOLOGY-ASSISTED ASSESSMENT APPROACHES

While the majority of studies, which have reported the use of technology-assisted assessment solutions have been promising and have demonstrated their feasibility in children, there have been few which have formally tested their use in the clinical setting. Such work has however been done in the adult post-stroke field, where robot-assisted solutions for assessment and rehabilitation have become increasingly commonplace (27, 55). The validity of objective motor behavioral measures was demonstrated by Bosecker and colleagues (56) who found a high correlation between kinematic and kinetic metrics collected using a robot fitted to the arm, and an extensive battery of standard clinical assessments of upper-limb motor function in a relatively large group (111) of chronic adult stroke patients. Furthermore, robotic tools have been shown to have sufficient sensitivity to detect significant differences in objective measures of arm use (such as task completion time, movement overlap, and phase difference) when performing

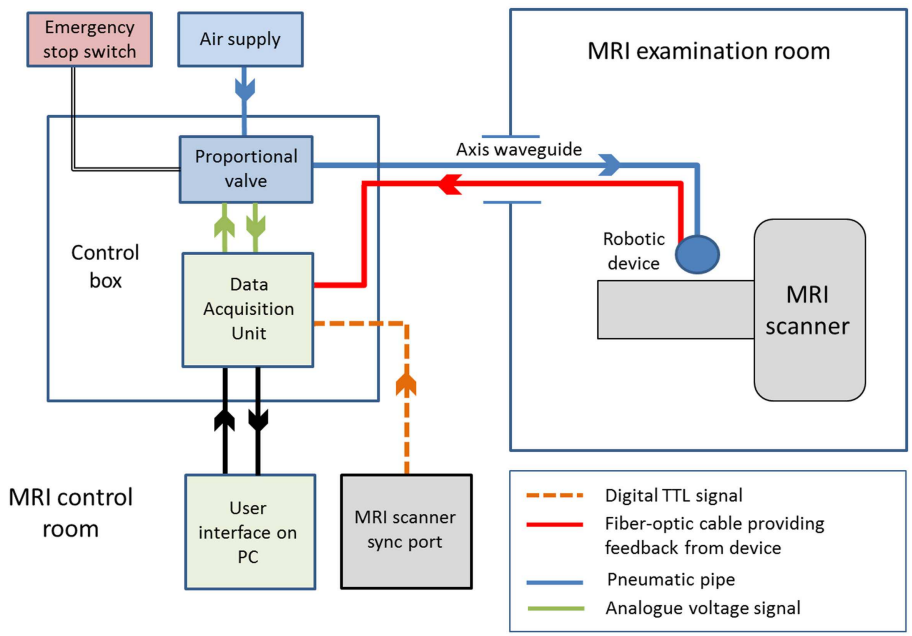

FIGURE 2 | Schematic diagram of an fMRI compatible robotic device control system. The robotic device can be controlled remotely via a control box located in the MRI control room. Actuation of the device can be achieved via timed opening of the proportional valve, which allows pressurized air through the pneumatic connection (running through the axis waveguide) to the device in the MRI examination room. Complete control of the system is achieved via a user interface running on PC software, and integration of the multimodal information through a data acquisition unit.

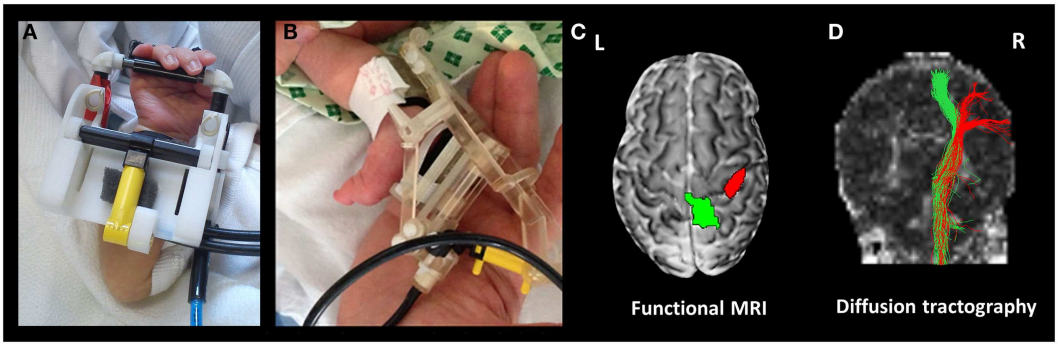

FIGURE 3 | Functional magnetic resonance imaging compatible devices can be used to precisely map functional activity and axonal pathways. The devices are fitted to the subjects' limbs prior to scanning, and can provide a safe and reproducible pattern of stimulation, which is fully automated and synchronized with fMRI data acquisition. Shown are devices fitted to the wrist **(A)** and ankle **(B)**. In a preterm infant at 35 + 4 weeks post-menstrual age, this approach can then be used to identify localized clusters of functional response **(C)** using fMRI (green cluster identified with passive movement of the left ankle, and red following passive movement of the left wrist), and their underlying structural connections can be delineated using diffusion tractography **(D)**.

an everyday task (drinking and pouring) with as few as 10 control subjects and 7 stroke patients (57). A clear difficulty inherent to studies of this population is the heterogeneity of patient groups, which may explain the apparent contradiction of a recent meta-analysis of upper-limb robot-assisted rehabilitation, which found a significant improvement in upper-limb motor function but no clear change in scores of activities of daily living (55).

Of the previously described clinical assessment tools, by nature of its application and off-line scoring, perhaps the most amenable to combination with a technology-based solution for infants is GM assessment. Indeed, studies have shown that the predictive power of GM assessment is preserved even when the assessment is automated by fitting motion tracking sensors to the extremities of the infants (in addition to video recording the infant), and then performing motion feature extraction on a highly filtered motion image using custom computer software (58–60). The sensitivity and specificity of GM assessment for the prediction of cerebral palsy has also been found to be greatly enhanced by combination with MRI at term equivalent age (61).

In comparison to other clinical assessment tools and commonly used neuroimaging modalities (such as CrUSS), MRI at term equivalent age has a relatively good evidence base supporting its use for the prediction of later cerebral palsy (15). It has become the diagnostic investigation of choice in infants who have suffered brain injury at the time of birth, and can provide vital prognostic information through precise delineation of both the acquired lesion and its associated effects. In addition to standard anatomical images (usually T1 and T2 weighted), it is also worthwhile acquiring a diffusion weighted image (DWI), particularly for identifying areas of early ischemia before it can be readily seen on structural images. Following perinatal stroke, MRI has been shown to be highly predictive of later unilateral spastic cerebral palsy (in the side opposite to the brain injury) if abnormal signal is seen in the ipsilesional posterior limb of the internal capsule (PLIC), thalamus, and perirolandic cortex (62); and if pre-Wallerian degeneration is evident on DWI in the contralesional cerebral peduncle (63). Similarly following Hypoxic Ischemic Encephalopathy (HIE), a number of studies have demonstrated that abnormal signal in the basal ganglia and thalami is highly predictive of dyskinetic or athetoid cerebral palsy (64, 65); injury to the basal ganglia, thalami, and brainstem are predictive of feeding and communication difficulties (66), and changes in myelination of the PLIC predictive of adverse motor outcome (67). Of further interest, a meta-analysis of the predictive power of MRI techniques for predicting adverse outcome following HIE found that abnormal deep gray matter lacate/*N*-acetyl-aspartate (NAA) ratio on single proton MRS (magnetic resonance spectroscopy) has the highest pooled sensitivity (68).

The predictive power of MRI may be further enhanced by the application of acquisition sequences, which can also visualize other diverse aspects of brain tissue composition, microstructure, and function. A clear example of this is tractography derived from diffusion MRI data, which utilizes information about the random diffusion of water inside the brain to delineate the major axon fiber bundles within the brain's white matter (69). Using this approach, it is possible to further increase the sensitivity of MRI for the prediction of later cerebral palsy by identifying subtle asymmetry in the microstructural integrity of the corticospinal tracts in infants with focal brain injury (70, 71). When correlated with neurological or developmental outcome, these measures may therefore provide additional prognostic information, and can also be used as highly accurate cerebral biomarkers for studies of pathological effects and treatments (72).

A key feature of fMRI is that it can visualize functional activity in the whole brain, thereby allowing the mapping of large-scale functional networks including connectivity to physiologically important deep brain structures such as the thalamus, basal ganglia, and cerebellum (73). Moreover, spatial information about the localization of functional brain activity derived from fMRI experiments can be combined with that derived from other analysis techniques such as diffusion MRI tractography, thereby allowing a detailed visualization of the macroscopic framework of both functional and structural connectivity (74). While it may not be feasible to perform the aforementioned fMRI studies in the

standard clinical setting, it is likely that the results acquired in specific specialist centers may provide dramatic new insights about the pathophysiological processes underlying the development of later cerebral palsy, which will therefore be of relevance to all working in the field. This notion becomes even more compelling, when it is considered that there have been a number of case reports of children in which dramatic sustained alterations in functional neuroanatomy have been seen following focal brain injury earlier in life (75–77). fMRI and advanced imaging techniques such as tractography may therefore present an accurate means with which to characterize and monitor neural (re)organization and neuroplasticity following brain injury, and furthermore to longitudinally monitor how they may be influenced by activity and therapeutic intervention (78).

Despite its prominent place in current clinical practice, there is however a surprising paucity of clinical studies, which have investigated the early predictive value of structural MRI for later cerebral palsy in other high risk populations (in particular prematurely born infants born without evidence of focal brain pathology) [for recent review see Ref. (79)]. While normal CrUSS has been found to confidently predict a normal motor outcome later in childhood, MRI at term equivalent age has a high predictive sensitivity for cerebral palsy but suffers from poor specificity, especially in infants with moderate cerebral abnormalities (80, 81). This limitation may be due to small study population sizes (and in particular the relatively small number with adverse outcome) leading to wide confidence intervals. It may also represent limitations inherent to the imaging techniques (such as restricted spatial resolution or contrast), the unpredictability of later childhood influences on neurodevelopment, or the lack of longer term diagnostic information (81). With the increasing availability of MRI, the development of MR compatible incubators and specialized physiological monitoring equipment, it will likely become possible to resolve this uncertainty (82, 83).

SUMMARY

With the aim of guiding and assessing early intervention strategies, an ongoing goal must be to develop accurate assessment tools, which are capable of providing enhanced diagnostic and prognostic power at an earlier developmental stage during infancy. Future assessment tools should not only have an increased specificity and sensitivity for predicting cerebral palsy, but could also be predictive of the severity of later cerebral palsy (rather than just its occurrence). The integration of sensor- and robot-assisted techniques into both clinical and neuroimaging assessment may allow the collection of detailed measures of motor function and development, thereby giving clinicians much needed patient-specific information on their outlook, recovery and therapeutic effectiveness.

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UCH-L1 and GFAP serum levels in neonates with hypoxic–ischemic encephalopathy: a single center pilot study

Martha V. Douglas-Escobar[1,2]*, Shelley C. Heaton[3], Jeffrey Bennett[4], Linda J. Young[5], Olena Glushakova[6], Xiaohui Xu[7], Daphna Yasova Barbeau[1], Candice Rossignol[1], Cindy Miller[1], Alissa M. Old Crow[4], Ronald L. Hayes[6] and Michael D. Weiss[1]

[1] *Department of Pediatrics, University of Florida, Gainesville, FL, USA*
[2] *Department of Pediatrics, University of California San Francisco, San Francisco, CA, USA*
[3] *Department of Clinical and Health Psychology, University of Florida, Gainesville, FL, USA*
[4] *Department of Radiology, University of Florida, Gainesville, FL, USA*
[5] *Department of Statistics, University of Florida, Gainesville, FL, USA*
[6] *Banyan Biomarkers, Inc., Alachua, FL, USA*
[7] *Department of Biostatistics, University of Florida, Gainesville, FL, USA*

Edited by:
Anna Purna Basu, Newcastle University, UK

Reviewed by:
William Doster Watson, Uniformed Services University of the Health Sciences, USA
Vincenzo Salpietro, Chelsea and Westminster Hospital, UK

****Correspondence:***
Martha V. Douglas-Escobar, Department of Pediatrics, University of California San Francisco, 505 Parnassus Avenue, Moffitt Room M 680, San Francisco, CA 94143, USA
e-mail: martha.douglas-escobar@ucsf.edu

Objective: We examined two potential biomarkers of brain damage in hypoxic–ischemic encephalopathy (HIE) neonates: glial fibrillary acidic protein (GFAP; a marker of gliosis) and ubiquitin C-terminal hydrolase L1 (UCH-L1; a marker of neuronal injury). We hypothesized that the biomarkers would be measurable in cord blood of healthy neonates and could serve as a normative reference for brain injury in HIE infants. We further hypothesized that higher levels would be detected in serum samples of HIE neonates and would correlate with brain damage on magnetic resonance imaging (MRI) and later developmental outcomes.?

Study Design: Serum UCH-L1 and GFAP concentrations from HIE neonates ($n = 16$) were compared to controls ($n = 11$). The relationship between biomarker concentrations of HIE neonates and brain damage (MRI) and developmental outcomes (Bayley-III) was examined using Pearson correlation coefficients and a mixed model design.

Result: Both biomarkers were detectable in cord blood from control subjects. UCH-L1 concentrations were higher in HIE neonates ($p < 0.001$), and associated with cortical injury ($p < 0.055$) and later motor and cognitive developmental outcomes ($p < 0.05$). The temporal change in GFAP concentrations during (from birth to 96 h of age) predicted motor developmental outcomes ($p < 0.05$) and injury to the basal ganglia and white matter.

Conclusion: Ubiquitin C-terminal hydrolase L1 and GFAP should be explored further as promising serum biomarkers of brain damage and later neurodevelopmental outcomes in neonates with HIE.

Keywords: biomarkers, HIE, UCH-L1, GFAP

INTRODUCTION

Hypoxic–ischemic encephalopathy (HIE) is a serious birth complication due to systemic asphyxia (1), which occurs in about 20 of 1,000 full-term infants and nearly 60% of very low-birth weight (premature) newborns (2–4). Between 10 and 60% of babies who exhibit HIE die during the newborn period (4). Of the surviving neonates with HIE, up to 25% have permanent neurodevelopmental handicaps in the form of cerebral palsy (CP), mental retardation, learning disabilities, or epilepsy (5–7). Until recently, treatment of HIE consisted of supportive care including respiratory support, treatment of hypotension, careful monitoring of fluid and electrolytes, and treatment of seizures. In the last decade, research has shown that therapeutic hypothermia improves the neurological and neurodevelopmental outcome of a subgroup of infants with moderate HIE (8–11). Since, more than 47% of treated infants are non-responders to hypothermia (10), we should strive for a better patient stratification including time, location and severity of brain lesion. To be effective, hypothermia should be initiated as soon as possible and no later than 6 h after the initial insult (9, 12). Unfortunately, the bedside clinician is not currently able to accurately identify the neonate who will respond versus the non-responder because accurate clinical indicators cannot be assessed during treatment due to sedatives administered and the effects of hypothermia itself. Therefore, the development of a new, rapid, and reliable prognostic test is essential for making therapeutic decisions.

Abbreviations: GFAP, glial fibrillary acidic protein; HIE, hypoxic–ischemic encephalopathy; UCH-L1, ubiquitin carboxyl-terminal esterase L1.

Current monitoring and evaluation of HIE, outcome prediction, and efficacy of hypothermia treatment rely on a combination of a neurological exam, ultrasound, magnetic resonance imaging (MRI), and electroencephalography (EEG) (13–17). However, these methods do not adequately identify hypothermia non-responders. MRI requires transport of the neonate with a requisite 40–45 min scan, which is not appropriate for unstable neonates. The amplitude integrated EEG (aEEG), is a helpful bedside monitoring technique for seizures and predict HIE outcomes. However, hypothermia depresses the aEEG and thus limits its early predictive ability. Improvement in aEEG tracings may be delayed until the patient is rewarmed and no longer on sedation (18, 19). Consequently, the development of a simple, inexpensive, non-invasive, rapid biochemical test is essential to identify severity of brain injury, distinguish responders from non-responders to hypothermia and assess outcome.

Although many potential biomarkers of brain damage exist, glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase L1 (UCH-L1) hold significant promise in this population. *GFAP* is a type III intermediate filament that forms part of the cytoskeleton of mature astrocytes and other glial cells but is not found outside the CNS (20). CNS injury that causes gliosis and subsequently up-regulates GFAP makes GFAP an attractive candidate biomarker for brain injury screening. *UCH-L1*, a highly abundant neuronal protein, is thought to play a critical role in cellular protein degradation during both normal and pathological conditions (21). Both pre-clinical and clinical studies showed that UCH-L1 levels were elevated in CSF and serum following TBI and stroke in a manner significantly associated with measures of injury severity and outcome (22–28). A recent pilot study in neonates with HIE found that serum GFAP but not UCH-L1 correlated with motor outcomes (29).

In this study, we examined the levels of GFAP and UCH-L1 in cord blood to establish normative levels in cord blood. We next examined the level of UCHL-1 and GFAP in patients with HIE undergoing hypothermia at several time points. We hypothesized that the serum concentrations of these two select proteins would be (1) detectable in cord blood in neonates, (2) the levels of UCH-L1 and GFAP would be elevated in the patients with HIE compared with controls, (3) higher in neonates with HIE undergoing hypothermia who had worse brain MRI, and (4) higher HIE patients with a worse developmental outcomes (Bayley-III). Comparisons were then made between the volume of injury as measured by MRI and neurodevelopmental outcomes as measured by formal developmental testing.

MATERIALS AND METHODS

PATIENT POPULATIONS

All aspects of this study were approved by the University of Florida Institutional Review Board, and patients were enrolled after obtaining the informed consent from the parents, at Shands Teaching hospital at the University of Florida Health (2012–2013). All studies were approved by the Institutional Review Board at the University of Florida.

Control population

Cord blood samples were obtained from healthy neonates who did not have any prenatally diagnosed known risk factors for HIE. The neonates had Apgar scores of 8 or higher at 1 min and 8 or higher at 5 min. In addition, none of the controls had abnormal physical examination or were admitted to the NICU.

HIE population

Patients with HIE who were eligible for hypothermia therapy were recruited. Entry criteria for hypothermia included a gestational age of 35 weeks or greater, birth weight of 1.8 and $\leq$6 h of age. The neonates had evidence of encephalopathy as defined by seizures or abnormalities on a modified Sarnat exam (level of consciousness, spontaneous activity, posture, tone, primitive reflexes including suck and Moro, autonomic system findings including pupils, heart rate, and respirations). Evidence of hypoxic–ischemic injury as defined by a pH of 7.0 or less and/or a base deficit of $<$16 or a pH between 7.01 and 7.15 and/or a base deficit between 10 and 15.9 or no blood gas available and an acute perinatal event (cord prolapsed, heart rate decelerations, uterine rupture) (**Table 1**).

BLOOD SAMPLE PROCESSING

Blood (1 ml) was collected using a tiger top 3.5 ml serum separator tube (BD Vacutainer SST Plus Blood Collection Tube). Samples were allowed to clot upright at room temperature for 30 min in processing lab (45 $\pm$ 15 min from time of collection), then spun at 1200 RCF (g) at room temperature for 15 min if fixed angle centrifuge rotor. Spun serum was then collected and transferred using a disposable transfer pipette into a 2 ml cyrovials with red cap inserts (USA Scientific REF 1420-9705). A fiberboard cryogenic storage box (Fisher Part No. 03-395-114 or equivalent) was used to store spun serum aliquots. The samples were then stored in −80°C freezer. The samples were stored (until all samples were collected) and then hand-carried in dry ice, from our laboratory to Banyan Biomarkers to be process immediately upon arrival.

ENZYME-LINKED IMMUNOSORBENT ASSAY

Blinded serum samples were processed at Banyan Biomarkers, Incusing proprietary sandwich enzyme-linked immunosorbent assays (ELISAs) to determine the concentrations and temporal

Table 1 | The patient demographics of the 16 subjects with HIE are shown.

Gestational age	**38 ± 2 weeks**
Transferred	44%
NRFHT	55%
Apgar scores	
5 min	2 ± 2
10 min	3 ± 2
Intubation in DR	81%
Cord pH	6.98 ± 0.16
Base deficit	−18 ± 6
Sarnat stage	
Moderate	41%
Severe	58%
Inotropic support	50%
EEG seizures	19%

profiles of UCH-L1 and GFAP in human serum. Banyan has successfully used these sensitive biomarker assays in a series of previously published studies in adults following TBI and epilepsy (24–26, 30–32). A detailed description of the ELISA procedures has been published elsewhere (31). Briefly, both mouse monoclonal capture antibody against recombinant UCH-L1 full length and partial protein, and rabbit polyclonal detection antibody were produced in-house at Banyan Biomarkers, Inc. Similarly, proprietary mouse monoclonal antibody for solid phase immobilization and a polyclonal rabbit detection antibody were used for ELISA to detect the levels of intact GFAP and its breakdown products. Such an approach allows more sensitive detection of GFAP analytes from patients' blood (31, 33). Standard curves using recombinant proteins were generated for each assay and quantitative determination of the biomarker levels in unknown samples were based on four-parameter non-linear regression analyses using SigmaPlot software (Systat, Chicago, IL, USA).

MRI SCORING AND VOLUMETRIC ANALYSIS

Magnetic resonance imaging was performed between 4 and 12 days of age since the majority of the patients are stable enough for transport. All patients were scanned on the same 3 T scanner (Verio; Siemens, Erlangen, Germany), with a 32-channel head coil. Analysis focused on the T1-weighted, T2-weighted, and diffusion weighted (DWI) abnormalities. A single subspecialty board-certified neuroradiologist with 10 years of experience in neonatal imaging interpreted all the MRI images using the Barkovich scoring system (34). Brain injury was stratified according to location into four groups: cortical, basal ganglia and thalamus, deep white matter. The volumetric T1-weighted images (3D MP-RAGE), with effective voxel size of 1 mm × 1 mm × 1 mm were analyzed using ITK-SNAP Version 2.0 (Penn Image Computing and Science Laboratory). While correlating with DWI and standard T1- and T2-weighted images, the area of abnormality was manually traced on each slice. The volume of abnormality was then calculated automatically by the software.

STATISTICAL ANALYSIS

All statistical analyses were performed using SAS 9.3 (Cary, NC, USA). To compare levels of UCH-L1 and GFAP either between HIE and control neonates or HIE neonates over time, a generalized linear model was fit using a logarithmic link function so that the assumption of normality of the residuals was approximately met. This provides a comparison of medians on the data scale. In addition, the differences in variability among neonates over time and the correlation among measurements from the same neonate were accounted for in the modeling process. The Pearson correlation coefficient was used to assess the association between each of the protein biomarkers (UCH-L1 and GFAP) and percent injury in the cortex, white matter, and basal ganglia regions as measured by MRI and the cognitive, language, and motor developmental outcomes. Receiver–operator characteristic (ROC) curves were constructed to determine area under the curve (AUC) for each serum biomarker value obtained from each of the time points sampled to with the ability to detect HIE. The graphs were created using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

DEVELOPMENTAL TESTING

Neurodevelopmental outcome of the HIE infants was assessed between 4.8 and 10 months of age using the Bayley Scales of Infant and Toddler Development, Third Edition (35). The three primary Bayley-III Index Scores (cognitive, language, and motor) were used to classify HIE participants into "good outcome" and "poor outcome" groups. All raw scores were transformed into norm-referenced standard scores (scale mean = 100 with SD = 15) using the Bayley-III scoring software published with the test. Standardized scores that were at or >1 SD below the normative sample mean (i.e., scores ≤ 85) were classified as indicative of "poor outcome" (i.e., developmental delay in one or more domains).

RESULTS

UCH-L1 AND GFAP IN UMBILICAL CORD BLOOD

A total of 11 patients had cord blood collected. Both UCH-L1 and GFAP were able to be detected in the serum samples from cord blood (**Figures 1A,B**).

HIE SUBJECTS

The demographics of the HIE patients are shown in **Table 2**. A total of 16 subjects underwent hypothermia and had serum samples obtained. A total of 54 samples were obtained for analysis.

RECEIVER–OPERATOR CHARACTERISTIC

We analyzed the ability of UCH-L1 and GFAP to detect HIE using values measured at different time points (**Figure 2**). The ROC plots showed that UCH-L1 measured from 0 to 6 h after the birth had AUC = 1.00, and there is a decreasing trend of AUC with the time of measurement. AUC summarizes diagnostic accuracy, with those approaching 1.00 being very accurate while AUC approaching 0.5 are considered more associated with pure chance. The AUC for GFAP increased slightly over time, with all point estimation >0.5.

CONCENTRATIONS OF UCH-L1 AND GFAP IN SERUM OF NEONATES WITH HIE COMPARED WITH CORD BLOOD AT 0–6 H AFTER BIRTH

The serum levels of UCH-L1 at 0–6 h of age ($n = 4$) were compared with age-matched controls ($n = 11$). The results demonstrate that UCH-L1 levels are significantly higher in the HIE group compared with the controls ($p < 0.001$). Notably, the lowest concentration in the HIE patients was 18 ng/ml compared to the highest value of 4.8 ng/ml in the controls (**Figure 1A**). The levels of GFAP were not significantly elevated compared to control cord blood samples at 0–6 h of age ($p = 0.7$) (**Figure 1B**).

SERUM CONCENTRATIONS OF UCH-L1 AND GFAP OVER TIME NEONATES WITH HIE

The serum concentrations of UCH-L1 were still above control concentrations at 12 h ($p < 0.05$). By 24 h, there was no difference between the control concentrations and the HIE concentrations. The concentration of UCH-L1 dropped significantly between the 0–6 h and 12 h sampling time points. The 0–6 h sampling time point was significantly higher than all other sampling time points (**Figure 3A**). The serum concentrations of GFAP demonstrated a trend over time with a rise in the concentration over the 96 h measured (**Figure 3B**).

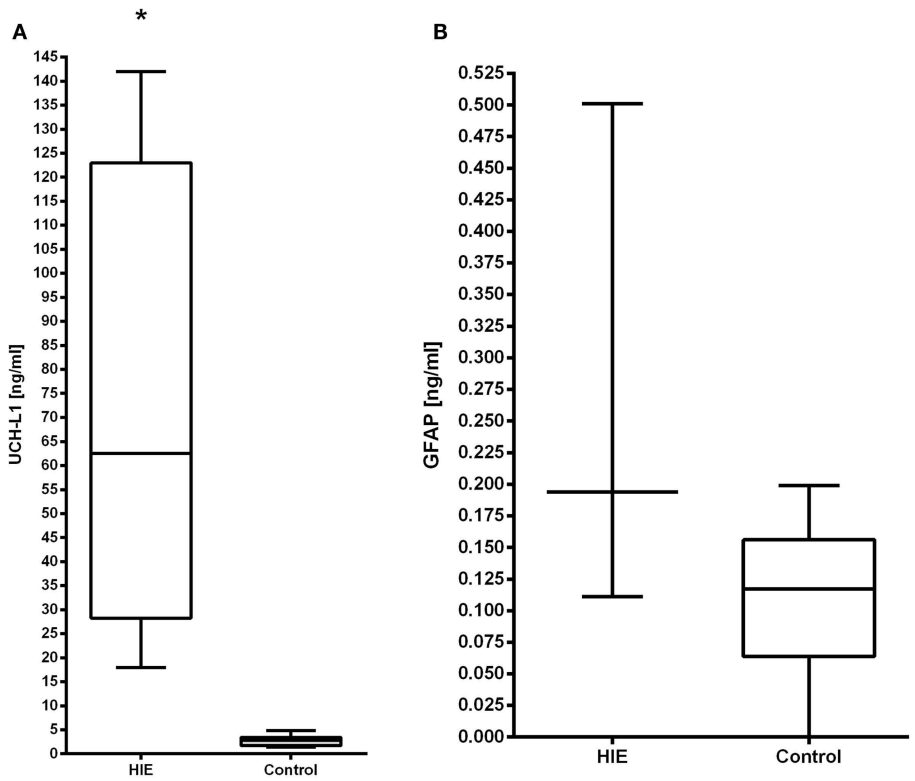

FIGURE 1 | The serum concentrations of UCH-L1 (A) and GFAP (B) in neonates with HIE are compared with control neonates. Increased serum levels of UCH-L1 at 0–6 h in HIE patients ($n = 4$) compared with controls ($n = 11$), $p < 0.0001$. The serum concentration of GFAP did not differ statistically from the control population.

Table 2 | Demographic and key medical variables in a prospective sample of HIE neonates treated with hypothermia who had serial biomarker samples obtained and formal developmental follow-up.

Patient	Biomarker profile						Neurodevelopmental outcome				
	UCHL (ng/ml)			GFAP (ng/ml)			(Bayley-III assessment)				
	0–6 h	12 h	24 h	0–6 h	12 h	24 h	Age (months)	Cognitive	Language	Motor	Outcome
Subject 1	141.71	–	10	0.2	–	0.06	9.9	130	106	82	Poor
Subject 2	–	–	1.792	0	–	NA	10	135	121	100	Good
Subject 3	17.82	2.281	2.308	0	0.09	0.1	9.7	105	100	94	Good
Subject 4	–	–	1.296	–	–	0.03	5.5	145	91	79	Poor
Subject 5	–	59.43	–	–	0.065	–	6.1	85	103	46	Poor
Subject 6	66.18	36.65	15.96	0.501	0.369	0.218	4.8	85	83	82	Poor

Bayley-III index scores (scale mean = 100; SD = 15); –, samples were unavailable.

CORRELATION OF SERUM UCH-L1 AND GFAP CONCENTRATIONS AND MRI IN NEONATES WITH HIE

The serum concentrations of GFAP demonstrated the strongest correlations with the percent injury of the cortex at a time of 0–6 h of age ($p = 0.08$) and the percent injury of white matter and basal ganglia injury at 12 h of age ($p = 0.06$). UCH-L1 concentrations were associated with cortical injury at 12 h ($p = 0.055$).

CORRELATION OF SERUM UCH-L1 AND GFAP CONCENTRATIONS AND DEVELOPMENTAL OUTCOMES IN NEONATES WITH HIE

Developmental outcomes were performed on 6 subjects ranging in age from 4.8 to 10 months of age with an average age of 8 ± 3 months (**Table 2**). Four of the six subjects had poor developmental outcomes defined as performance on any of the primary Bayley-III domains (motor, cognitive, and language) that was at

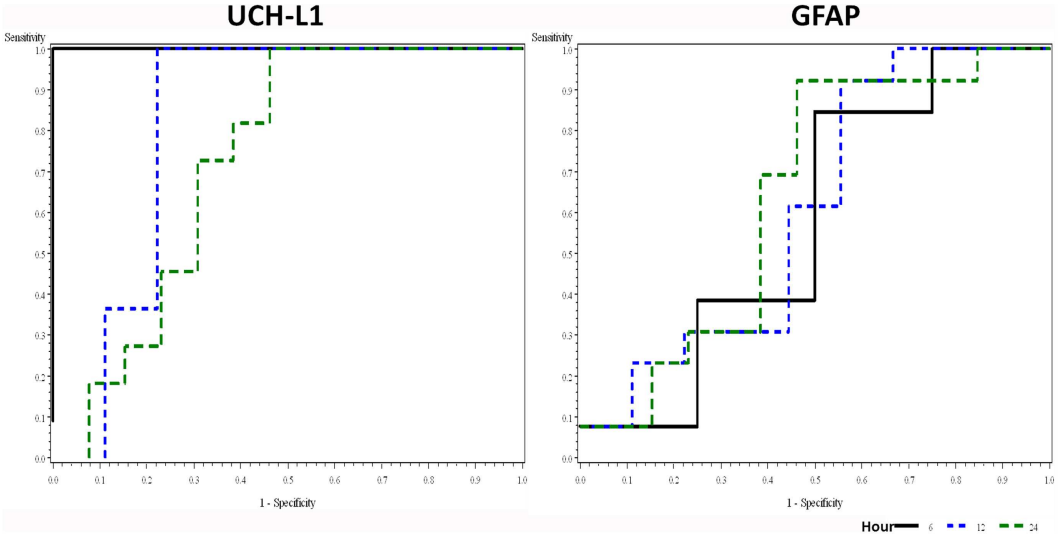

FIGURE 2 | Receiver–operator characteristic plots of UCH-L1 and GFAP at different time point in detecting HIE. Area under curve (AUC with 95% confidence interval) for UCH-L1 is 1.00, 0.83 (0.57–1.00), and 0.73 (0.51–0.94) for 0–6, 12, and 24 h, respectively. AUC (95% CI) for GFAP is 0.58 (0.15–1.00), 0.61 (0.33–0.88), and 0.64 (0.41–0.87) for 0–6, 12, and 24 h, respectively.

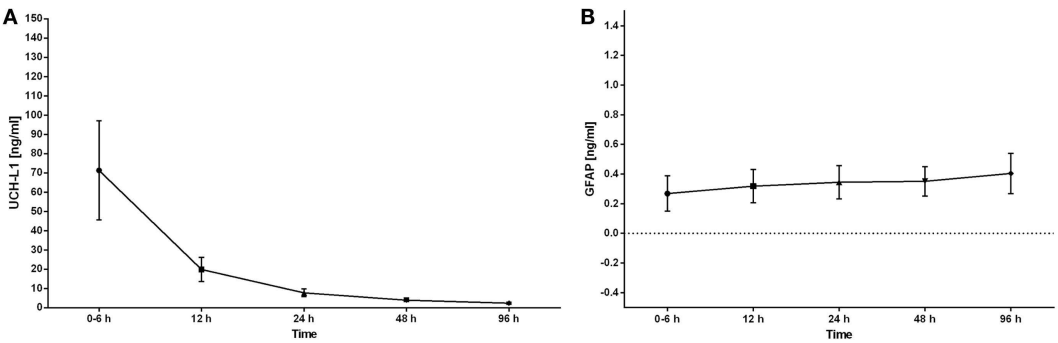

FIGURE 3 | The serum concentrations of UCH-L1 and GFAP in neonates with HIE are plotted over the time of sample collection. The serum concentrations are expressed as the mean ± SEM. The serum concentration of UCH-L1 decreased rapidly over the initial 24 h with the highest concentrations obtained at 0–6 h **(A)**. The serum concentrations of GFAP increased over the 96 h of sampling **(B)**.

least 1 SD lower than age-matched normative data. All four subjects who were classified as having poor developmental outcomes exhibited delays in motor development. Two of the four subjects had additional delays in cognitive development, and one subject exhibited delays in all three developmental domains (motor, cognitive, and language).

The UCH-L1 profiles in the patient who had a good outcome demonstrated a serum concentration of 17.82 ng/ml at 0–6 h of age with a decrease in the concentration to 2.28 ng/ml at 12 h (**Figure 4A**). Subjects with a poor developmental outcome had a mean serum concentration of UCH-L1 103 ng/ml at 0–6 h with a decrease to 48 ng/ml at 12 h (**Figure 4A**). The mean concentration at 24 h was 8.62 for the subjects with a poor developmental outcome and 2.05 in the subjects with a good prognosis.

The concentration of GFAP in the patient who had a good outcome was undetectable at 0–6 h with an increase to 0.092 ng/ml at 12 h. Subjects with a poor outcome had a mean serum concentration of GFAP at 0–6 h of 0.348 ng/ml with a decrease to 0.217 ng/ml at 12 h (**Figure 4B**).

Subject 6, who exhibited developmental delays across all three domains assessed, had serum concentrations of UCH-L1, which were persistently elevated over the first 24 h. The subject's concentrations of GFAP were also elevated and were the highest measured values over the first 96 h (**Table 2**).

Ubiquitin C-terminal hydrolase L1 concentrations at 12 h were correlated with developmental motor outcomes ($p < 0.05$). Further, the temporal changes in UCH-L1 were predictive of both developmental motor ($p < 0.05$) and cognitive outcomes ($p < 0.05$). Finally, the temporal change in the concentration of GFAP was predictive of developmental motor outcomes ($p < 0.05$).

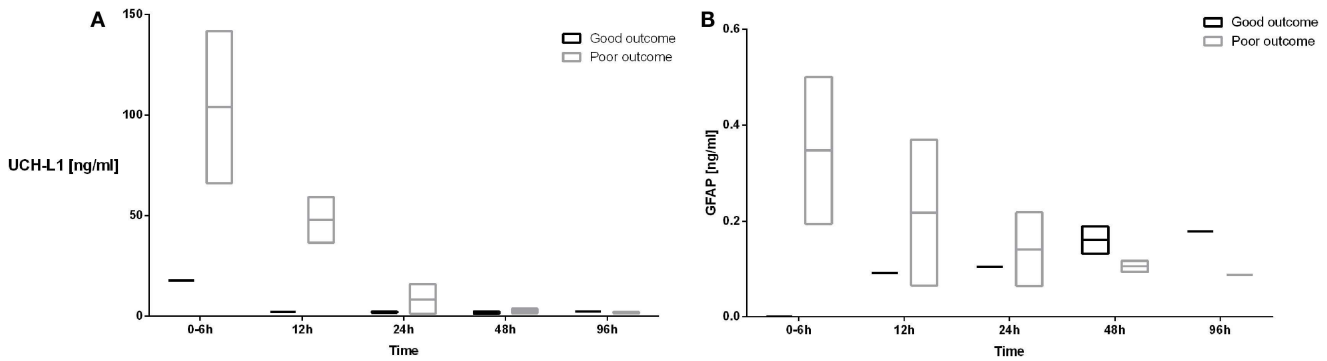

FIGURE 4 | The serum concentrations of UCH-L1 (A) and GFAP (B) in neonates with HIE who had good (black boxes and lines) and poor developmental outcomes (gray boxes and lines) are plotted over the 96 h sampling period. The mean serum concentrations are represented by the line while the bar represents the minimum and maximum serum concentrations.

DISCUSSION

Hypothermia has become standard of care for neonates with HIE. As the field of neonatal neuroprotection evolves, there must be a method to distinguish neonates who will respond to hypothermia to those whom hypothermia will not benefit. A rapid bedside test offers the greatest promise to objectively stratify these neonates. In this report, we have described two proteins, UCH-L1 and GFAP, which are candidates to potentially utilize for stratification. To the best of our knowledge, this is the first report, which has correlated serum biomarkers temporal changes in biomarker concentrations with developmental outcomes.

As a clinical tool, the earlier the proteins can be identified, the better the potential clinical utility. Both proteins were able to be detected in umbilical cord blood samples. Umbilical cord blood measurements were chosen because it is readily obtainable in healthy term neonates and most closely matched the 0–24 h sampling points in our HIE population. The umbilical cord blood provided a true control since the neonates all went to the newborn nursery and did not have serum samples routinely obtained for clinical indications in the first 6 h post birth. Umbilical cord blood may provide information about the degree of injury at the time of birth and potentially the timing of injury based on the serum concentrations at the time of birth. In the future, our group plans to obtain umbilical cord blood from neonates with HIE. The normative information obtained in this study will be used for comparison.

The serum concentrations of UCH-L1 were significantly elevated in the neonates with HIE compared with the control population. Importantly, for the bedside clinician, the elevation in UCH-L1 concentrations occurred at 0–6 h of age and continued to be higher than control concentrations for the first 24 h of sampling. The concentrations of UCH-L1 at 12 h correlated with developmental motor outcomes in neonates with HIE. We suspect that the concentrations at 0–6 h would also predict motor outcomes, but too few samples were obtained to make any firm conclusions. UCH-L1 is an abundant protein localized exclusively to the perikarya and dendrites of neurons (36). Therefore, UCH-L1 may be a very important early marker of neuronal injury. UCH-L1 is resistant to endogenous brain and serum proteases (37). These characteristics along with our results make UCH-L1 an ideal candidate to serve as a biomarker of brain injury in neonates with HIE.

The temporal change in the concentration of UCH-L1 was shown to correlate with developmental motor and cognitive outcomes. Specifically, there was a rapid decrease in the serum concentration in the two neonates with HIE who had good developmental outcomes. This demonstrates that UCH-L1 may be a candidate biomarker to stratify neonate undergoing hypothermia as responders versus non-responders.

The serum concentrations of UCH-L1 at 12 h demonstrated a weak correlation with cortical injury. Our previous work demonstrated that UCH-L1 serum concentrations were higher in neonates with evidence of basal ganglia injury on MRI. It is important to note that these studies were performed in neonates who had not undergone hypothermia therapy. Previously, UCH-L1 and GFAP were shown to have elevated concentrations in neonates with HIE undergoing hypothermia, which correlated with severe MRI abnormalities or death (38).

The blood brain barrier is an anatomic structure composed of brain capillary epithelium joined by tight junctions and the foot processes of astrocytes (39). The blood brain barrier prevents the passive movement of water-soluble molecules larger than 500 Da (39). Following HIE, the blood brain barrier becomes permeable with severe disruption in severe HIE (40). The permeability of the blood brain barrier is measured by CSF to plasma albumin ratios, which may be difficult to perform in an unstable neonate following HIE (40). Serum UCH-L1 has been shown to be a marker of the integrity of the blood brain barrier in patient with traumatic brain injury (39). Therefore, the elevation of biomarker may provide information for the delivery of large neuroprotective drugs, which do not typically cross the blood brain barrier. This may provide a bedside test to determine the exact windows for drug administration, which will individualize care for each patient.

Glial fibrillary acidic protein is a cytoskeleton intermediate filament protein of the astrocytes and is released into the blood following astrocyte death (41). In this report, GFAP concentrations were not higher than controls at 0–6 h but had a higher mean. The concentrations of GFAP in cord blood and at 0–6 h

obtained in our study were similar to those previously reported in neonates with HIE undergoing hypothermia (29). Although the concentrations of GFAP were not different than the control samples at 0–6 h, it is important to note that the temporal change in the concentration of GFAP was predictive of developmental motor outcomes.

The serum concentrations of GFAP demonstrated a strong correlation with injury to the cortex (0–6 h), basal ganglia, and white matter (12 h) as detected by MRI. A previous study demonstrated correlations between GFAP concentrations and MRI injury in serum samples obtained at 12 and 24 h of life (38). Taken together, our results along with those published suggest that GFAP may be an important biomarker in predicting regions of brain injury in the first 24 h of life in neonates with HIE.

This study has demonstrated that UCH-L1 is elevated as early as 0–6 h in patients with HIE and the concentrations correlate with developmental motor outcomes. Our data differ from Chalak et al. (42) that found no correlation between UCH-L1 and developmental outcomes, and no temporal changes in UCH-L1 (during hypothermia) (42). Our data demonstrated that the temporal profile of UCH-L1 correlated with the developmental motor and cognitive outcomes. It is possible that our results are different because we had neonates with more severe HIE, making more feasible to demonstrate motor outcomes differences between the groups. Our study's major weakness was a small number of patients. However, this has been a weakness of all biomarker studies in neonates, to date (43). In addition, the developmental outcomes were performed at a multiple ages post-injury. Although this could be construed as a weakness, this is the only the second study, which has demonstrated that serum based biomarkers correlate with long-term neurologic outcomes and further suggests that serum biomarkers may be used to predict long-term outcomes (42). Furthermore, outcomes were evaluated using norm-referenced scoring methods that control for any inherent differences due to varied outcome time points across subjects. The results from this study provide further data to support the use of UCH-L1 and GFAP in a larger study to evaluate the correlation between serum concentrations and outcomes at 18–24 months of age (42). UCH-L1 appears to offer great promise as a serum based bedside marker to be utilized by the bedside clinician managing neonates with HIE.

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Insulin-like growth factor receptor signaling is necessary for epidermal growth factor mediated proliferation of SVZ neural precursors *in vitro* following neonatal hypoxia–ischemia

Dhivyaa Alagappan, Amber N. Ziegler, Shravanthi Chidambaram, Jungsoo Min, Teresa L. Wood† and Steven W. Levison*†

Department of Neurology and Neuroscience, New Jersey Medical School, Rutgers University, Newark, NJ, USA

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Ashok K. Shetty, Texas A&M Health Science Center College of Medicine, USA
Hassan Azari, Shiraz University of Medical Sciences, Iran

****Correspondence:***
Steven W. Levison, NJMS-UH Cancer Center, 205 South Orange Avenue, Newark, NJ 07103-1709, USA
e-mail: steve.levison@rutgers.edu

†Teresa L. Wood and Steven W. Levison are co-senior authors.

In this study, we assessed the importance of insulin-like growth factor (IGF) and epidermal growth factor (EGF) receptor co-signaling for rat neural precursor (NP) cell proliferation and self-renewal in the context of a developmental brain injury that is associated with cerebral palsy. Consistent with previous studies, we found that there is an increase in the *in vitro* growth of subventricular zone NPs isolated acutely after cerebral hypoxia–ischemia; however, when cultured in medium that is insufficient to stimulate the IGF type 1 receptor, neurosphere formation and the proliferative capacity of those NPs was severely curtailed. This reduced growth capacity could not be attributed simply to failure to survive. The growth and self-renewal of the NPs could be restored by addition of both IGF-I and IGF-II. Since the size of the neurosphere is predominantly due to cell proliferation we hypothesized that the IGFs were regulating progression through the cell cycle. Analyses of cell cycle progression revealed that IGF-1R activation together with EGFR co-signaling decreased the percentage of cells in G1 and enhanced cell progression into S and G2. This was accompanied by increases in expression of cyclin D1, phosphorylated histone 3, and phosphorylated Rb. Based on these data, we conclude that coordinate signaling between the EGF receptor and the IGF type 1 receptor is necessary for the normal proliferation of NPs as well as for their reactive expansion after injury. These data indicate that manipulations that maintain or amplify IGF signaling in the brain during recovery from developmental brain injuries will enhance the production of new brain cells to improve neurological function in children who are at risk for developing cerebral palsy.

Keywords: cell proliferation, stem cell niche, growth factors, central nervous system, regeneration

INTRODUCTION

Many studies over the past decade have touted the regenerative potential of the endogenous neural precursors (NPs) of the subventricular zone (SVZ) following injury. Although encouraging, the extent of cell replacement, both in terms of the numbers of cells produced and the variety of cell types generated is limited. However, by understanding the mechanisms that regulate the growth of SVZ cells, therapeutic strategies can be designed to achieve a more significant level of regeneration after CNS injury. In an earlier study, we used flow cytometry on SVZ cells isolated acutely after neonatal hypoxia–ischemia (H–I) and showed that epidermal growth factor receptor (EGFR) expression increased significantly on primitive NPs of the SVZ during recovery from neonatal H–I. Furthermore, we showed that pharmacologically inhibiting the EGFR reduced the expansion of the NPs that normally occurs (1).

Despite wide acceptance that the EGF receptor is a key mitogen for NPs, there is increasing evidence that other ligands are central regulators of NP cell cycle progression. Two key regulators are the insulin-like growth factors (IGF-I and IGF-II). The critical importance of IGFs for neural development was shown in studies that produced genetically engineered mice with disrupted IGF-I gene expression. These gene targeted mice showed profound *in utero* and postnatal growth retardation (2). IGF-II knockouts showed similar growth retardation with onset earlier during gestation and, like the IGF-1 deficient mice, showed a 40% reduction in body mass at birth (3). Homozygous IGF-1R null mice have more profound developmental deficits at the end of gestation, and they do not survive beyond a few hours after birth (2).

Complementary studies using transgenic mice where IGF-I was expressed downstream of the regulatory elements for nestin that are specifically active in NPs (4), have provided additional insights into the roles of IGF-I in CNS neural development. In these studies, overexpressing IGF-I increased the fraction of embryonic day 14 neuroepithelial cells in S phase by over 15% compared to wild type animals. When these transgenic mice were analyzed postnatally, there was a significant increase in the total number of neurons generated accompanied by a 26% reduction in the number of apoptotic cells compared to age matched wild type animals.

These results suggested that, *in vivo*, IGF-I promotes the proliferation of NPs during the genesis of the CNS and that IGF-I also promotes survival (5).

In vitro studies on the effects of IGF-I on NPs have been somewhat contradictory. Arsenijevic et al. (6) showed that embryonic day 14 murine striatal NPs failed to proliferate in serum free, insulin free media even when supplemented with EGF and FGF-2. However, upon IGF-I addition, they grew effectively and formed neurospheres in culture. IGF-I stimulation as short as 24 h, in the constant presence of EGF, was sufficient to produce neurospheres comparable in number to those generated during continuous incubation with IGF-I and EGF. This effect could not be recapitulated by either a transient or continuous co-incubation of IGF-I with FGF-2. Therefore, the authors suggested that the continuous presence of IGF-I is not necessary for EGF-stimulated NSC proliferation (6). However, the results of that study were contradictory to those of Aberg et al. (7), who studied adult rat hippocampal NPs (7). Aberg et al. (7) showed that upon FGF-2 pretreatment in the presence of a low concentration of insulin [100 ng/ml], the expression of IGF-1R increases. They described distinct proliferative effects of FGF-2 (without IGF-I addition) and IGF-I (without FGF-2 addition) and showed that the effects were additive when both FGF-2 and IGF-I were used together (7).

The apparent contradiction between these studies, apart from the distinct cell types used, could be due to the presence of insulin in the culture medium, which will activate the IGF receptor system. As the majority of NP studies use a superphysiological concentration [25 μg/ml] of insulin in the culture medium that activates IGF-1R signal transduction, the role of IGF-1R signaling is generally unappreciated. Specifically, in postnatal NPs additional studies are needed to assess the separate effects of IGF-1R signaling from EGF receptor signaling. Moreover, if the IGFs increase the proliferation and survival of NPs, then it is important to evaluate the role of the IGFs in the context of developmental brain injuries where disturbances in IGF signaling may perturb normal brain development or recovery from injury. Thus, the focus of this study was to test the hypothesis that IGF-1R is necessary for the expansion of NPs following brain injury in the presence of EGF.

MATERIALS AND METHODS

NEONATAL HYPOXIA–ISCHEMIA

All animal work was approved by the institutional animal care and use committee guidelines of the New Jersey Medical School. Timed pregnant Wistar rats (Charles River Laboratories, Charles River, DE, USA) were housed on a 12 h light/dark cycle at 25°C with Purina rodent chow (catalog #5001). Following a normal delivery, the litter size was culled to 12 pups per litter. Neonatal H–I was induced on postnatal day 6 (P6) rat pups [P0 as day of birth] by a permanent right common carotid artery [CCA] cauterization followed by systemic hypoxia. Briefly, P6 rat pups were anesthetized with isoflurane [5% induction and 3% maintenance] following which a midline neck incision was made. The right CCA was separated from the vagus nerve and ligated using a bipolar cauterizer (Malis bipolar cauterize and bipolar cutter, Codman, Randolph, MA, USA) at 10 V. The skin incision was sutured using 4-0 silk, and the pups were returned to the dam for 2 h. Prior to hypoxia, the pups were pre-warmed in jars partially submerged in a 37 C water bath for 20 min after which rat pups were subjected to systemic hypoxia in jars with humidified 8% O_2/92% N_2 for 75 min. Sham-operated control animals had their right CCA separated from the vagus nerve but it was not ligated, and they were subjected to hypoxia. Following the hypoxia, the animals were left in the jars for 15 min at normoxia after which they were returned to their cages.

PRIMARY NEUROSPHERE ASSAY

After 3 days of recovery from H–I, P9 pups were decapitated, their brains removed, and neurospheres generated essentially as previously described (8). For experiments on neurospheres from untouched rats, P4 rat pups were used. Under aseptic conditions, a cut was made 2 mm from the anterior pole of the brain. A second cut was made approximately 3 mm posterior to the first cut. The hippocampus, corpus callosum, and the meninges were removed under the microscope. Using forceps, 12 o'clock and 3 o'clock incisions were made, and the region enclosed between the cortex and the ventricle containing the SVZ was removed and placed in fresh PGM [1 mM $MgCl_2$, 0.6% Dextrose in PBS, pH 7.3]. The tissue was mechanically dissociated using forceps and then enzymatically by the addition of 0.05% Trypsin/EDTA at 37°C for 7 min. The trypsin was inactivated by the addition of an equal volume of newborn calf serum, and the tissue was resuspended in ProN media [DMEM/F12 1:1 media containing 10 ng/ml d-biotin, 25 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 5 ng/ml selenium, 50 μg/ml apo-transferrin, 50 μg/ml gentamycin], and triturated in proN. The triturated suspension was passed through a 40 μM Nitex screen, and the cells were collected by centrifugation at 200 × *g* for 2 min and washed with ProN. The cells were counted with 0.1% Trypan blue dye under a hemocytometer and plated at 5×10^4 cells/ml in ProN media containing 25 ng/ml insulin and supplemented with 2 ng/ml EGF, 1 ng/ml FGF-2, 15 ng/ml IGF-I, 28 ng/ml IGF-II, or combinations as stated. The cells were cultured at 37°C in 5% CO_2 incubators and fed every 2 days by removing approximately half of the media and replenishing with fresh media.

SECONDARY NEUROSPHERE PROPAGATION

Primary neurospheres were collected from 12-well plates after 6 days *in vitro* and pelleted by centrifugation at 12,000 rpm for 5 min. The neurospheres were enzymatically dissociated for 5 min at 37°C in a 0.01% trypsin/EDTA solution in GHCKS buffer (11 mM glucose, 20 mM HEPES, 10 mM citrate, 4 mM KCl, 110 mM NaCl, and 0.002 g/l phenol red). Trypsin was inhibited by adding 10% FBS in ProN media. For spheres that were generated for the early growth response factor-1 (EGR-1) experiment, Accutase was used to dissociate the primary spheres. The spheres were dissociated by trituration in ProN media using progressively less media and smaller pipette tips. The number of viable cells was determined in a hemocytometer via exclusion of 0.1% trypan blue dye. The cells were plated into a plastic 12-well tissue culture plate at a density of 5×10^4 viable cells/ml in ProN media supplemented with growth factors as per experimental conditions. Cell cultures were fed every 2 days by removing approximately half the media and replacing with an equal volume of fresh media.

NEUROSPHERE QUANTIFICATION

A neurosphere was defined as a free-floating cluster of at least 25 μm in diameter. Prior to counting the spheres, the plates were shaken to ensure uniform distribution of the spheres in the well. The number of neurospheres in five random fields under 4× was determined for each well. The total number of neurosphere producing cells, the NPs in the population, was extrapolated from the average number of spheres per field, area of the field, and area of the well. The cells were plated at 5×10^4 cells/ml and at 2 ml/well in a 6-well tissue culture dish for each condition. For neurosphere volumetric measurements, digital photographs were captured from at least 10 neurospheres from each condition ($n = 6$ SVZ per condition) and volumes calculated using IP Lab 3.6 software.

PROPIDIUM IODIDE LABELING FOR FLOW CYTOMETRY ANALYSIS OF CELL CYCLE

Neurospheres were dissociated by enzymatic and mechanical dissociation in 0.05% trypsin-EDTA, fixed in 70% ethanol, and then stored at −20°C until analysis. Cells were incubated with RNase 1 (Sigma, St. Louis, MO, USA) for 15 min and then stained with 50 mM propidium iodide (PI). PI is a fluorescent dye that intercalates into DNA, thus, the DNA content of a cell correlates with fluorescence intensity, providing a profile of cells in different phases of the cell cycle. PI stained cells were characterized using a Becton Dickinson FACS scan and the data acquired using CellQuest™ software (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using ModFit™(Verity Software House, Topsham, ME, USA). ModFit uses a robust automatic analysis engine to detect peak and to identify ploidy patterns that correspond to different phases of the cell cycle.

WESTERN BLOT ANALYSES

Total cell lysates from neurospheres were washed in ice-cold PBS and isolated in sodium dodecyl sulfate (SDS) buffer (62.5 mM Tris–HCl, 2% SDS, 10% glycol, 50 mM DTT, 1/100 protease inhibitor cocktail, 1 mM Na3VO4, and 1 mM NaF). Lysates were briefly sonicated and then subjected to a protein assay (Bio-Rad, Hercules, CA, USA). A total of 15–30 μg of cell lysates were boiled at 100°C for 5 min and resolved on 7, 10, or 4–12% mini gels by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were electrotransferred to nitrocellulose membranes and blocked in 5% milk in TBS-1% Tween buffer for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C (1:500, pHistone 3; 1:5000, β-actin; 1:250 for all other antibodies). The following day, membranes were washed 3 × 5 min in TBS-1% Tween and incubated with secondary antibodies, HRP-conjugated goat anti-mouse or goat anti-rabbit antibodies (1:5000) for 1 h at room temperature. The detection of HRP-conjugated secondary antibodies was performed with ECL (Perkin Elmer, Boston, MA, USA) using the Ultra-LUM imaging device (Claremont, CA, USA).

QUANTITATIVE REAL-TIME PCR

RNA was isolated and reverse transcribed to cDNA using the iScript kit from Bio-Rad. Q-PCR was performed on the samples as described in Ref. (9) using beta actin as the internal control and with Quantitect real-time primers for EGR-1 (QT00385546).

RESULTS

THE INCREASE IN NEUROSPHERE NUMBER AND SIZE FOLLOWING NEONATAL H–I REQUIRES EGFR ACTIVATION TOGETHER WITH IGF-I CO-SIGNALING.

Our previous studies have demonstrated that there is an increase in the number and size of neurospheres generated from the SVZ following H–I injury. This increase in neurosphere size and number occurs in response to EGF but cannot be recapitulated by FGF-2 (1). The serum free media for SVZ NPs has superphysiological levels of insulin that can activate the IGF-1R in addition to the insulin receptor (IR). Considering the results of the studies reviewed in the Introduction together with our data, we hypothesized that IGF-1R is necessary for the expansion of NPs following brain injury in the presence of EGF. Therefore, we performed experiments to evaluate the necessity of IGF-1R signaling by lowering the insulin concentration in the media, to levels that only stimulate IR, from 25 μg/ml "high" to 25 ng/ml "low" in NPs when exposed to EGF, FGF-2, or both. We found that this manipulation reduced the number of neurospheres that were obtained from the ipsilateral hemisphere cultured in low insulin media compared to high insulin media in the presence of EGF by 2.5-fold (**Figure 1A**). Similarly, reducing the concentration of insulin in the medium affected the size of neurospheres. Neurospheres produced from the ipsilateral hemisphere in EGF containing "low" insulin media were 3-fold smaller than "high" insulin media [$p < 0.05$] (**Figures 2A,B**).

As earlier studies had shown that FGF-2 promoted NP proliferation, we evaluated the necessity of IGF-1R signaling in the FGF-2-mediated response of NPs following injury. Again, lowering the insulin concentration from 25 μg/ml to 25 ng/ml, reduced the number of neurospheres obtained from the ipsilateral hemisphere by 3-fold in the presence of FGF-2. However, it is important to note that the numbers of neurospheres generated in the presence of FGF-2 were significantly lower compared to EGF even in the presence of "high" insulin levels (**Figure 1B**). The size of neurospheres obtained in FGF-2-media was not different when cultured in the presence of 25 μg/ml "high" insulin or 25 ng/ml "low" insulin media (**Figures 2A,C**).

We had previously shown that neurospheres grow larger in size and number from the ipsilateral hemisphere compared to contralateral and sham-operated control SVZ in EGF supplemented media (8). Interestingly, the neurospheres obtained when grown in EGF were significantly larger even in the presence of 25 ng/ml "low" levels of insulin (**Figures 1A** and **2A,B**). By contrast, there were no significant differences in neurosphere number and size in FGF-2 supplemented media containing 25 ng/ml "low" insulin (**Figures 1B** and **2A,C**). These data cannot be attributed to differences in survival as NPs maintained in low insulin and EGF for 7 days retained the capacity to form neurospheres when transferred to high insulin containing medium and EGF (data not shown), and similar results are obtained using this same experimental design with mouse NPs (9).

IGF-II IS A BETTER MITOGEN THAN IGF-I FOR GROWTH AND SELF-RENEWAL OF NPs

Emerging data indicate that NPs express both the IGF-I receptor as well as the insulin receptor A isoform, through which IGF-II, but not IGF-I, signals. Therefore, to determine whether IGF-I and

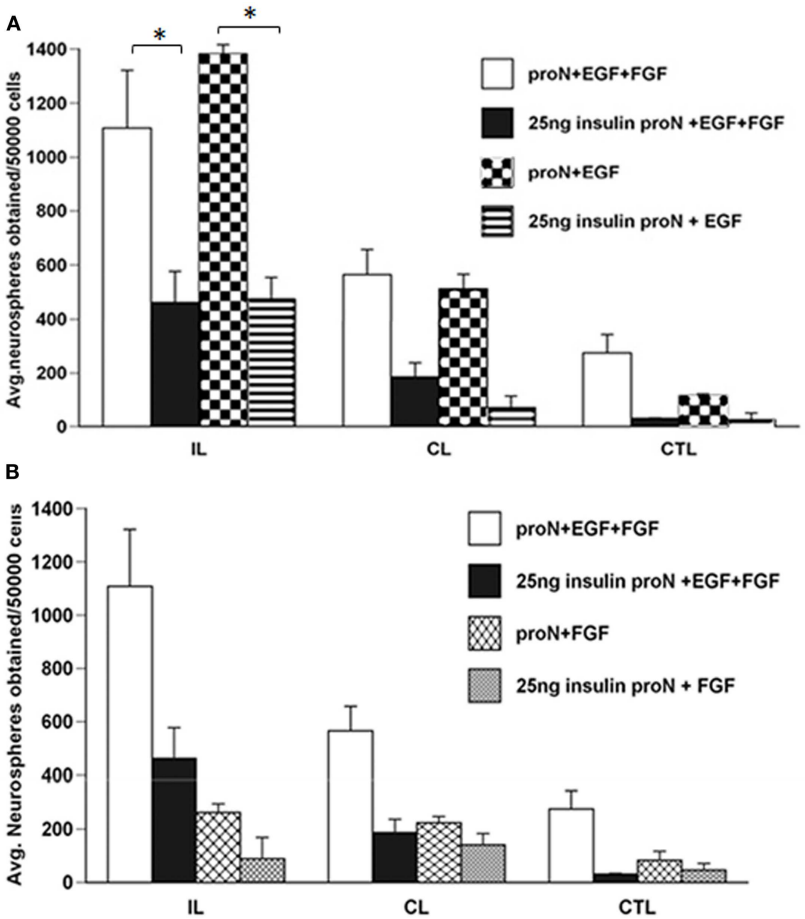

FIGURE 1 | The increase in neurosphere number after neonatal H–I requires EGFR activation together with IGF-R co-signaling. SVZ cells were isolated from ipsilateral (IL), contralateral (CL), and sham-operated control (CTL) at 3-day recovery from H–I. The dissociated cells were cultured in media containing 25 μg/ml "high" insulin or 25 ng/ml "low" insulin media supplemented with 2 ng/ml EGF **(A)** or 1 ng/ml FGF-2 **(B)** for 7 days. The numbers of neurospheres obtained per 50,000 cells plated was counted. $^*p < 0.05$ by MANOVA and Tukey's *post hoc* tests. Data represent the mean number of neurospheres ± SEM from three experiments with six animals per experiment.

IGF-II affect SVZ NP proliferation and self-renewal differently, we dissociated primary rat neurospheres and cultured them to generate secondary neurospheres in 25 ng/ml "low" insulin containing media supplemented with EGF and further supplemented with IGF-I alone, IGF-II alone, or the combination of IGF-I and IGF-II. IGF-I and IGF-II were used at their Kds for the IGF-1R. Supplementing the medium with IGF-I or IGF-II significantly increased the number of neurospheres generated vs. "low" insulin media with EGF. The combination of both IGF-I and IGF-II produced even greater growth than IGF-I alone (**Figure 3A**). Interestingly, IGF-II generated more ($p < 0.05$) neurospheres than IGF-I supplemented media. There was no significant increase between IGF-II and a combination of IGF-I and IGF-II on neurosphere numbers obtained (**Figure 3A**).

To establish whether these growth factors affected SVZ NP self-renewal independently or in combination, secondary neurospheres generated in 25 ng/ml "low" insulin containing media supplemented with EGF only or with IGF-I, IGF-II, or the combination were passaged and then maintained in 25 μg/ml "high" insulin media with EGF. Interestingly, spheres grown in IGF-II generated more tertiary spheres than spheres grown in IGF-I (**Figure 3B**). The number of tertiary spheres generated from cells exposed to IGF-I and EGF with "low" insulin was not significantly different from those exposed to "low" insulin media containing EGF alone in the absence of exogenous IGF-I (**Figure 3B**). In previous studies, we evaluated how the IGF ligands affected sphere potentiality. Spheres that were propagated in EGF containing medium supplemented with either IGF-I, IGF-II, or IGF-I + IGF-II were differentiated and stained for markers of neurons or glia. Spheres propagated in IGF-I and EGF were rarely tripotential. By contrast, the percentage of tripotential neurospheres was greatest in IGF-II supplemented medium (9).

IGF-1R AND EGFR CO-STIMULATION IS NECESSARY FOR CELL CYCLE PROGRESSION

To determine whether cooperatively stimulating the IGF and EGF receptors was necessary for cell cycle progression, and thus the expansion of the population in culture, we assessed cell cycle using

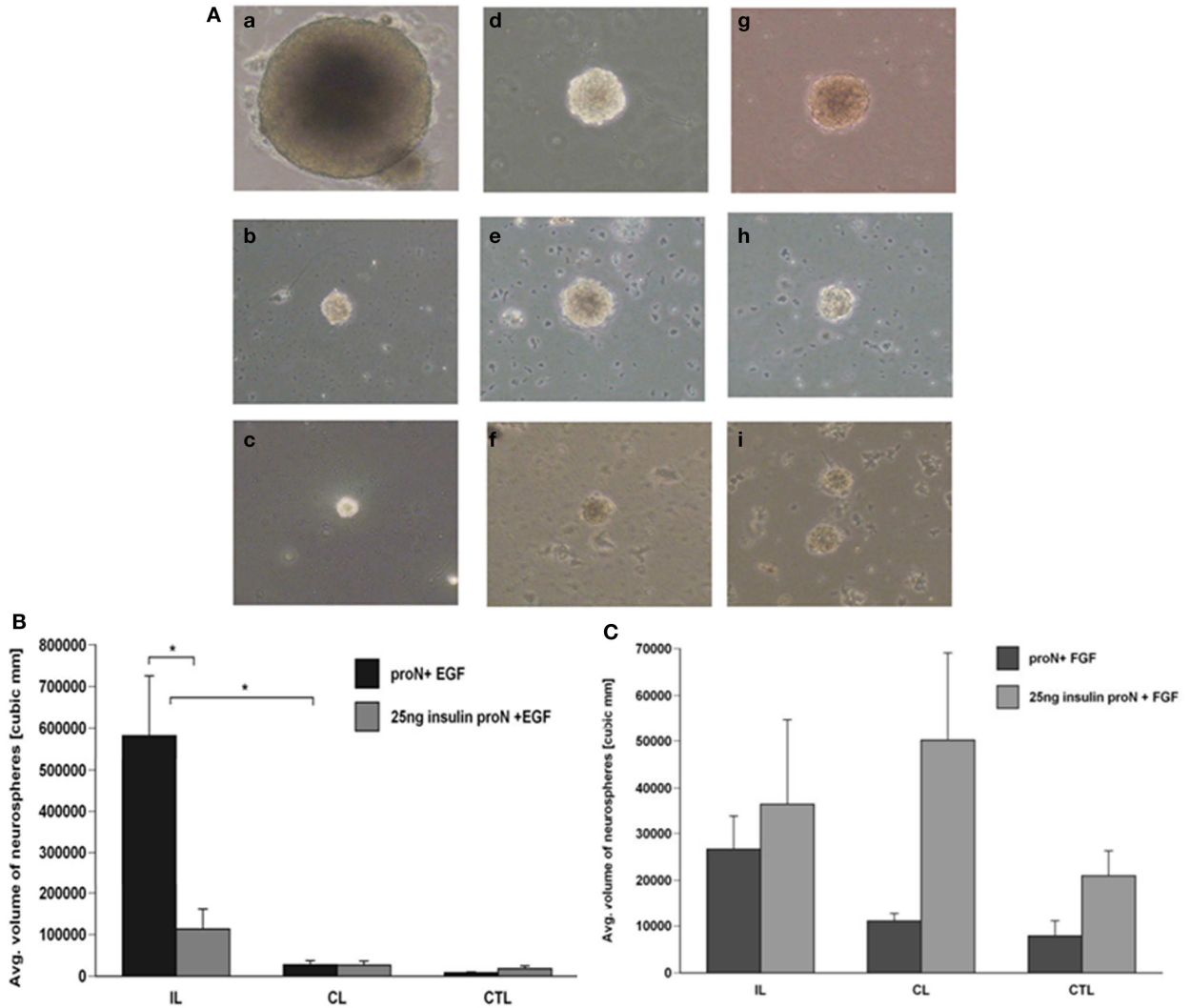

FIGURE 2 | Neurospheres grow larger in the presence of IGF-R signaling together with EGF but not FGF-2. **(A)** Neurospheres were generated from ipsilateral (a–c), contralateral (d–f), and sham-operated control (g–i) SVZ at 3-day recovery from H–I and cultured in 25 μg/ml insulin["high" insulin proN] supplemented with 2 ng/ml EGF (a, d, g); 25 ng/ml insulin["low" insulin] supplemented with 2 ng/ml EGF (b, e, h) or 1 ng/ml FGF-2 (c, f, i) for 7 DIV. **(B)** The average number of neurospheres obtained per 50,000 SVZ cells was quantified for EGF in "high" and "low" insulin. **(C)** Quantification of the average size of neurospheres obtained with EGF in "high" and "low" insulin from ipsilateral (IL), contralateral (CL), and control hemispheres (CTL). $*p < 0.05$ by MANOVA and Tukey's *post hoc* tests. Data represent mean number/size of neurospheres + SEM from three experiments with six animals per experiment.

flow cytometry. Secondary neurospheres were propagated for 3 DIV in "high" insulin media with EGF. The neurospheres were then starved for 18 h and stimulated for 72 h with either "low" insulin or "high" insulin, with or without EGF. An analysis of cells in S, G1, and G2 phases of the cell cycle using PI showed that there was a significant decrease in percentage of cells in G1 only when both IGF-1R and EGFR were stimulated (**Figure 4A**). There was a trend toward an increase in the proportion of cells in both S and G2 phases in the same, "high" insulin with EGF condition (**Figures 4A,B**). The variability in the number of cells found in S and G2 phases likely reflects the rapidity with which cell traverse these phases in contrast to the time a cell spends in G1, even when normally cycling. Using NP cultures, it is impossible to synchronize the cells to better study these shorter cell cycle phases as for traditional 2D culture conditions.

Cell cycle progression was further analyzed using Western blot for several key cell cycle proteins (**Figure 5**). These analyses revealed a large increase in cyclin D1 when both IGF-1R and EGFR were stimulated compared to "high" insulin alone (IGF-1R stimulation) or "low" insulin with EGF (EGFR stimulation). These conditions also increased levels of phosphorylated Rb. Taken together, these results support the conclusion that IGF-1R and EGFR cooperated to promote G1 progression through the G1/S transition. An increase in phosphorylated histone 3 suggests

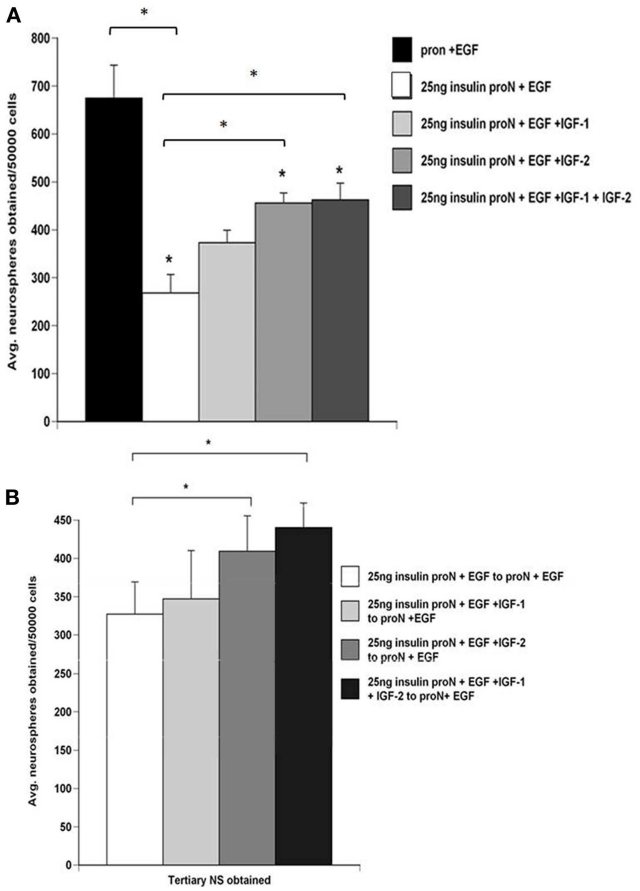

FIGURE 3 | NPs cultured in IGF-II generate more neurospheres which promotes their self-renewal better than spheres grown in IGF-I. SVZ cells from control rat brains were cultured in 25 μg/ml "high" insulin or 25 ng/ml "low" insulin media containing 2 ng/ml EGF supplemented with IGF-I (15 ng/ml), IGF-II (28 ng/ml), or both. **(A)** Quantification of the average number of neurospheres obtained per 50,000 SVZ cells. **(B)** Numbers of tertiary neurospheres obtained upon passaging to 25 ng/ml insulin media containing 2 ng/ml EGF. *$p < 0.05$ by MANOVA and Tukey's *post hoc* tests.

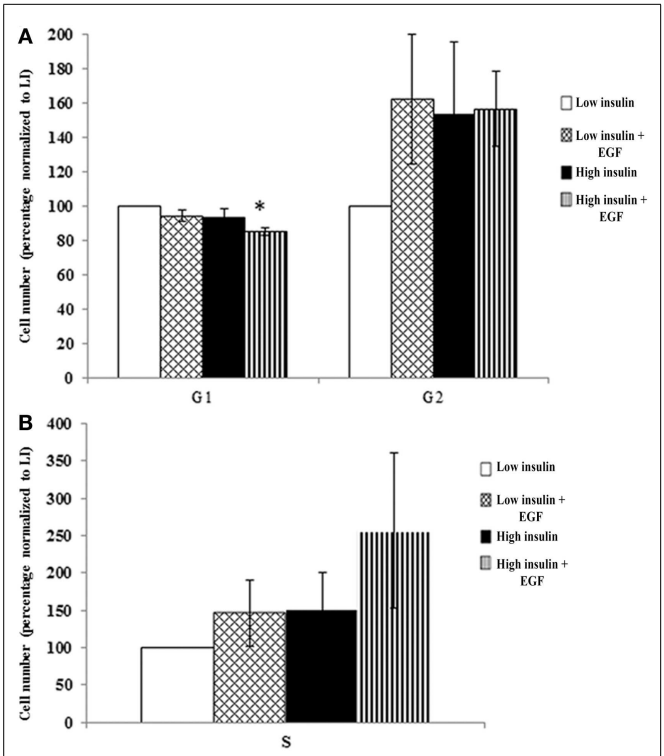

FIGURE 4 | IGF-1R activation together with EGFR co-signaling decreases G1 that leads to a trend in cell cycle progression. Neurospheres were cultured in "high" insulin media for 3 DIV, growth factor deprived for 18 h and then stimulated with either "low" or "high" insulin media with or without EGF for 72 h prior to cell cycle analysis using propidium iodide staining and flow cytometry. G1 and G2 **(A)** and S phase **(B)** were normalized to "low" insulin media ($n = 3$) averaged ± SEM, *indicates significance via ANOVA with Tukey's *post hoc*.

further progression into the G2 phase of the cell cycle. To further test whether the increase in phosphorylated histone 3, and cyclin D1 were dependent on the activation of the IGF-1R, we incubated the cells in an IGF-1R blocking antibody, A12, to block the action of "high" insulin or IGF-I on the IGF-1R (**Figure 5**). When A12 was present, both cyclin D1 and phosphorylated histone 3 levels decreased.

EGF AND IGF-I TOGETHER INCREASE LEVELS OF EARLY GROWTH RESPONSE FACTOR-1 (EGR-1)

EGR-1 regulates the expression of many genes including the EGF receptor. Therefore, we hypothesized that IGF-I might induce the expression of EGR-1 to promote EGF signaling. Neurospheres were grown in "high" insulin medium with EGF for 6–7 days and then passaged. The cells were then incubated in growth factor free medium for 16 h and then stimulated with EGF alone (no insulin), IGF-I alone (no insulin), both EGF and IGF-I (no insulin), EGF + "high" insulin or "high" insulin. The relative expression of EGR-1 was highest when both EGF and IGF-I were added to the media, however, contrary to our prediction, IGF-I alone only weakly induced EGR-1 expression (**Figure 6**) (Values averaged from two independent experiments).

DISCUSSION

It has been well-established that NPs can be cultured *in vitro* in the presence of EGF and/or FGF-2. Moreover, infusing these growth factors into the ventricles increases the proliferation of periventricular SVZ NPs (10, 11). Whereas, these two growth factors are widely viewed as necessary for neural development (10), the importance of the IGFs has been under appreciated, despite the fact that the IGFs are abundant in the immature brain where IGF-I is produced by neurons and IGF-II by the choroid plexus and meninges (12–14). Likely the IGFs have been ignored because the culture medium used to propagate NPs has high levels of insulin, which will stimulate IGF system receptors, thus, masking the essential roles of the IGFs and their receptors in NP cell biology.

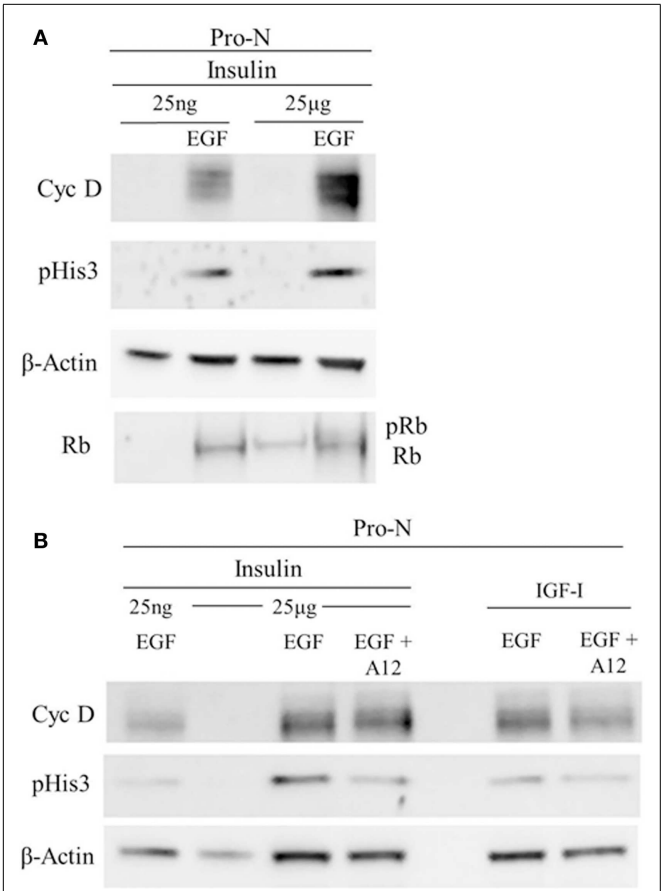

FIGURE 5 | Cell cycle progression marker expression is dependent on IGF-1R activation together with EGFR co-stimulation. Neurospheres were cultured for 3 DIV in "high" insulin, starved for 18 h, and then stimulated with "high" [25 μg/ml] insulin or "low" [25 ng/ml] insulin, with or without EGF **(A)** or with IGF-I with or without EGF **(B)** for 72 h. IGF-1R blocking antibody, A12, was used to block both IGF-I and "high" insulin stimulation of IGF-1R **(B)**.

Our data show clearly that the expansion of SVZ NPs following brain injury requires EGFR and IGF-1R co-signaling. Previously, we established that EGFR signaling is necessary for SVZ NP expansion following brain injury by pharmacologically inhibiting EGFR activation and effectively down-regulating the NP expansion *In vitro*. Furthermore, we showed that overexpressing a constitutively active EGFR within NPs was sufficient to increase entry of these cells into the S phase of cell cycle (1). But those studies were all conducted under conditions where there was concurrent IGF-1R stimulation. Here, we sought to determine the role of IGF-1R signaling in the EGF-mediated response of SVZ NPs. To do so, we replaced the supraphysiological levels of insulin [25 μg/ml] in our base culture media with a physiological concentration of insulin [25 ng/ml] that would activate the insulin receptor but not the IGF-1R, and we evaluated the effect of this manipulation on NP growth, survival, and self-renewal. We report that: (1) The increase in neurosphere number and size that occurs in response to neonatal H–I requires IGF-1R activation together with EGFR co-signaling; (2) NPs cultured in IGF-II generate more neurospheres and self-renew better than NPs grown in IGF-I; and (3) IGF-1R activation together with EGFR co-signaling stimulates cell cycle progression.

Reminiscent of our data, an earlier study by Lin et al. (15), showed that a single intraventricular injection of IGF-I immediately after neonatal H–I reduced the extent of damage and increased the number of proliferating cells within the SVZ (15). The fact that IGF-I was neuroprotective supports the view that beneficial effects of the IGFs in the context of neonatal H–I will extend beyond their effects on the NPs of the SVZ. For example, Wood et al. (16) demonstrated that infusing IGF-I into neonatal rat pups acutely after H–I increased the number of oligodendrocyte progenitor cells (OPC) in white matter (16).

Other studies conducted on primary OPCs are informative in helping to understand how two mitogens can promote cell cycle progression in NPs. These studies have revealed how two essential growth factors for the early OPCs, FGF-2 and IGF-I, can work synergistically to promote progression through cell cycle. In OPCs, FGF-2 activates p42/p44 to reduce the levels of the Cdk inhibitor p27 (Kip1), while IGF-I promotes the phosphorylation of GSK-3β via PI3-kinase to jointly increase cyclin D1 accumulation within the nucleus and to promote S phase transition. In our cell cycle analyses, IGF-1R stimulation with insulin decreased the percentage of cells in G1 with a trend toward an increase in S and G2. This correlated with an increased phosphorylation of Rb and increased levels of cyclin D1, indicators of G1 progression and essential for pushing cells past the G1/S transition. When IGF-1R activation was blocked using the A12 antibody, these alterations in cell cycle protein expression were inhibited indicating a need for receptor co-stimulation for cell cycle progression. Thus, it is likely that similar signal transduction mechanisms are in play in the NPs as in the OPCs. Similar studies on mouse NPs that compared NP growth when maintained in "high" insulin vs. IGF-II showed more progenitors in the "high" insulin group, indicating that the "high" insulin stimulates more progenitors to divide (17). Additional data are needed; however, mechanistic studies are difficult to complete using neurospheres because of the heterogeneity of the cell population in a neurosphere. Recent flow cytometry studies indicate that there are likely to be as many as 12 different NPs within a medium-sized neurosphere (18). This heterogeneity likely contributed to the failure to achieve a significant effect in the proportion of cells in S phase between groups.

Cross-talk between IGF-1R and EGFR has been reported in breast, lung, and prostate cancer cells (19, 20) as well as in COS cells (21), which has stimulated interest in understanding the cooperativity between these receptors. Roudabush et al. (21) showed that activating the IGF-I receptor lead to a matrix metalloprotease-dependent release of HB-EGF that caused EGFR transactivation. The transactivated EGFR accounted for IGF-I-stimulated recruitment of adaptor molecules such as Shc, Grb2, SOS, and the subsequent activation of the Ras/Raf/MEK/ERK signaling pathway (21). Similar signaling was implicated in the studies by Jones et al. (19). They reported that the initial growth arrest produced by an EGFR inhibitor gefinitib (ZD 1839; IRESSA) was lost within months of therapy in breast and prostate cell lines. The

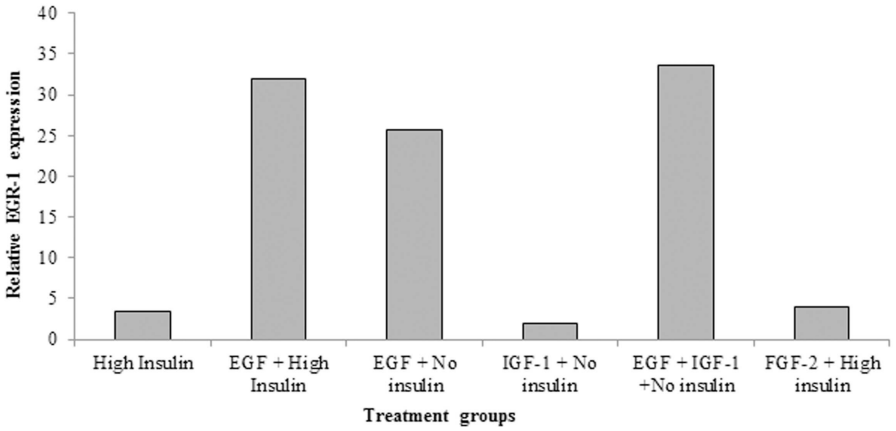

FIGURE 6 | EGF strongly induces EGR-1 expression in neural precursors. Primary neurospheres were generated from P4–5 pups in standard medium. After passaging, the cells were shifted to medium containing "High" insulin (25 μg/ml), EGF (2 ng/ml) + "high" insulin, EGF (2 ng/ml) + no insulin, IGF-I (15 ng/ml) + no insulin, or EGF (2 ng/ml) + IGF-I (15 ng/ml). Cells were grown for 6–7 days, whereupon total RNA was isolated and relative levels of EGR-1 mRNA measured by QRT-PCR. Values are the averages of two independent experiments.

acquired resistance to the drug was due to the formation of hybrid EGFR/IGF-1R receptors (19). Riedemann et al. (20) showed direct hybrid dimerization between the two receptors using reciprocal co-immunoprecipitation (20).

This study, together with our earlier studies, demonstrates that IGF signaling is important for the expansion of NPs observed after injury in response to EGF. NPs express both the IGF-1R and IR-A and our studies here on rat NPs and earlier studies on mouse NPs show that antagonizing the IGF-1R inhibits the growth of NPs (9). Additionally, IGF-1R stimulation with insulin results in neurospheres that contain more progenitors compared to ones cultured with IGF-II (17). We demonstrate that more neurospheres are generated in EGF containing media supplemented with IGF-II compared to IGF-I. While a combination of IGF-I and IGF-II is able to recapitulate the growth observed in "high" insulin media, our collective results from this paper and previous studies indicate that the spheres produced under these two sets of growth conditions are not comprised of the same cell populations as IGF-II exerts effects beyond those of IGF-1. Co-stimulation of EGFR and IGF-1R result in the "typical" growth that is observed in neurosphere cultures.

In a recent paper, Scafidi et al. (22) showed that stimulating the EGFR through intranasal heparin-binding EGF administration was both neuroprotection and pro-regenerative in that it enhanced the generation of new oligodendrocytes from progenitors and promoted functional recovery (22). They concluded that stimulating the EGFR in oligodendrocyte progenitors at a specific time after injury was a clinically viable treatment for babies born prematurely and who sustain white matter injury. However, their studies were conducted under conditions where the IGF-1R was activated. Though their results were impressive, it is possible that an even better outcome could have been achieved by also stimulating the IGF-1R. Thus, understanding the coordinated signaling between IGFs and EGFs may be essential to establish a robust regenerative response from SVZ NPs following brain injury.

AUTHOR CONTRIBUTIONS

Dhivyaa Alagappan: Conception and design, collection of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. Amber N. Ziegler: Conception and design, financial support, collection of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. Shravanthi Chidambaram: Conception and design, collection of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. Jungsoo Min: Conception and design, collection of data, data analysis and interpretation, manuscript writing, and final approval of manuscript. Teresa L. Wood: Administrative support, financial support, conception and design, manuscript writing, and final approval of manuscript. Steven W. Levison: Administrative support, financial support, conception and design, manuscript writing, and final approval of manuscript.

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Putative role of AMPK in fetal adaptive brain shut-down: linking metabolism and inflammation in the brain

Martin G. Frasch*[1,2]

[1] *Department of Obstetrics and Gynaecology, CHU Ste-Justine Research Center, Université de Montréal, Montreal, QC, Canada*
[2] *Department of Neurosciences, CHU Ste-Justine Research Center, Université de Montréal, Montreal, QC, Canada*
**Correspondence: mg.frasch@umontreal.ca*

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Irina Burd, Johns Hopkins University School of Medicine, USA
Suzanne Lee Miller, Monash University, Australia

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In a companion article of the same Research Topic, we present findings on the relationship of fetal adaptive brain shut-down and neuroinflammation during hypoxic acidemia (1). The findings are derived from the chronically instrumented non-anesthetized near-term ovine fetus model with and without chronic hypoxia (defined as arterial O_2Sat < 55%) subjected to umbilical cord occlusions (UCOs) of increasing severity. This model mimics human labor and is useful for studying the process of worsening acidemia that may precipitate perinatal brain injury. While the neuroinflammation overall decreases between 24 and 48 h post UCOs, the relationship between the degree of neuroinflammation and the timing of the adaptive brain shut-down reverses between these two time points, raising the question as to the underlying physiology.

We propose that adaptive brain shut-down in the fetus, evidenced by changes in EEG, may be mediated via adenosine monophosphate kinase (AMPK) signaling due to its controlling influence over cellular metabolism and interaction with inflammatory signaling pathways. By way of background, consider that the intracellular energy-sensor AMPK plays a key role in cellular metabolism, increases in cellular AMP/ATP ratio result in activation of AMPK via its phosphorylation (2, 3). pAMPK reduces ATP-consuming processes and promotes ATP-producing processes. Consequently, neuronal pAMPK decreases during the relatively less energy-consuming NREM sleep state, which is associated with increased EEG delta wave activity and ATP increase in adult rats (4). Fetal adaptive neuronal shut-down with worsening acidemia may also be mediated via adenosine A1 receptors (1, 5, 6). Notably, a combination of both A1 and AMPK signaling is also a plausible mechanism leading to adaptive brain shut-down. First, Gadalla et al. observed that 5-aminoimidazole-4-carboxamide riboside (AICA riboside), a compound with neuroprotective properties thanks to the AMPK activation, has an additional neuroprotective effect under metabolic stress via competition with adenosine for uptake by the nucleoside transporter leading to an increase of extracellular adenosine and subsequent activation of A1 receptors (7). Second, endogenous extracellular adenosine in physiological concentrations is, in turn, equally able to activate AMPK, an effect requiring active nucleoside transporters, such as CNT2 (8, 9). Both AMPK and A1 receptor activation result in suppression of the more energy-consuming glutamatergic excitatory synaptic neurotransmission (i.e., as opposed to GABAergic inhibitory signaling contributing only ~20% to the neuronal oxidative energy metabolism) (10, 11). Either way, the result would be a relative increase of intracellular ATP and decreasing AMPK levels.

Sag et al. demonstrated *in vitro* that AMPK signaling and pro-inflammatory mediators in macrophages are mutually coupled via negative feedback. AMPK suppresses pro-inflammatory responses such as lipopolysaccharide (LPS)-induced production of TNF-α and IL-6 and promotes macrophage polarization to an anti-inflammatory functional phenotype with increased production of IL-10 (12). Exposure of macrophages to pro-inflammatory cytokines increases AMPK dephosphorylation, while exposure to anti-inflammatory cytokines results in rapid AMPK phosphorylation, i.e., activation (12). Activation of toll-like receptor (TLR) 4 on macrophages by LPS and resultant NF-κB pathway activation lead to a loss of AMPK phosphorylation (13). Hence, the effects of AMPK on the regulation of inflammatory status indicate that the presence of AMPK and its activation are important to counteract inflammation. Similarly, *in vivo* AMPK is down-regulated in all immune cells during experimental autoimmune encephalomyelitis (EAE), the animal model of the autoimmune disease multiple sclerosis (14). Neuronal AMPK is widely expressed in the embryonic and adult rat brains *in situ* and promotes neuronal survival under conditions of hypoglycemia *in vitro* (15).

Adenosine monophosphate kinase activity and its anti-inflammatory consequences have been studied in the context of chronic hypoxia. Chronic hypoxia up-regulates pAMPK *in vitro* in healthy neonatal rat neuronal slice cultures, in the human glioblastoma cells and *in vivo* in the adult rats' pulmonary vasculature (7, 16, 17). Lactate is a principal energy source for neurons, especially in the developing brain (11, 18, 19). However, excess lactate within the extracellular space of the brain contributes to neuronal injury (3, 19). Recently, AMPK was also shown to play an important role in controlling the degree of

cellular inflammation in various cell types including glial cells, thus linking cellular metabolism and inflammation (2, 3, 20). Brain regional lactate acidosis increases neuronal intracellular pAMPK levels (21). At the same time, pAMPK also restricts microglial activation via the IFN-γ signaling pathway decreasing expression of STAT1-inducible inflammatory cytokines in adult mice (2) and, anti-inflammatory effects of AMPK activation have been demonstrated on NF-κB pathway in the primary glial cultures, notably from 1 to 3 days old rat pups, and *in vivo* in adult rats (22).

In seeming contrast to the above cited work, acute brain ischemia in adult male gerbils results in regional (CA1) transient pAMPK and lactate increases, ATP depletion, neuronal death, and microglial *activation* [as opposed to *suppression* of microglial secretory cytokine activity shown by Meares et al. and Giri et al. (3)]. These traits are reversed if an AMPK inhibitor is administered (3). Notably, these authors provided indirect evidence that cortical neuronal pAMPK increases within 90 min post insult, probably to compensate for lack of ATP, while the glial AMPK induction follows within 5 days, when neuronal death is observed. The seemingly contradictory findings regarding the effect of AMPK on neuroinflammation may result from the different animal models used (septic versus aseptic neuroinflammation), varying temporal profiles (acute versus chronic), and neuroinflammatory phenotyping (cytokine secretion versus cell morphology).

In light of the discussed AMPK physiology, it is intriguing to speculate that at 24 h post UCOs, AMPK-mediated neuronal shut-down correlates to decreased brain regional lactate levels, leading to a pronounced decrease in neuroinflammation. In contrast, at 48 h post UCOs, the relationship between the degree of neuroinflammation and the timing of the adaptive brain shut-down may be reversed due to several reasons. First, the fetal brain may be less capable of metabolizing lactate under conditions of pre-existing hypoxia with reduced metabolism and ATP reserves unable to sustain pAMPK activation beyond an acute response. Second, at 48 h post UCOs, regional lactate accumulation may have occurred, in addition to AMPK and inflammatory mediators. Third, one of the side effects of AMPK activation may be an increase in lactate production due to glycolysis, which may contribute to tissue injury (3). While future studies will have to validate these mechanisms in the perinatal brain, it seems plausible that lactic acidosis has the potential to induce variable degrees of microglial activation and neuronal shut-down in an AMPK-dependent manner, in chronically hypoxic fetuses with worsening acidemia. Further investigations are needed into the potential of intrapartum EEG–FHR monitoring to aid detection of adaptive brain shut-down to improve early postnatal diagnostic and therapeutic strategies, such as selecting at-risk newborns for hypothermic interventions to decrease cerebral metabolism (6, 23).

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Adaptive brain shut-down counteracts neuroinflammation in the near-term ovine fetus

Alex Xu[1†], Lucien Daniel Durosier[2†], Michael G. Ross[3], Robert Hammond[4], Bryan S. Richardson[1] and Martin G. Frasch[2]*

[1] Department of Obstetrics and Gynecology, Western University, London, ON, Canada
[2] Department of Obstetrics and Gynaecology and Department of Neurosciences, CHU Sainte-Justine Centre de Recherche, Université de Montréal, Montréal, QC, Canada
[3] Department of Obstetrics and Gynecology, LA BioMed at Harbor-UCLA Medical Center, Torrance, CA, USA
[4] Department of Pathology, Western University, London, ON, Canada

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Ravi Goyal, Loma Linda University, USA
Gavin John Clowry, Newcastle University, UK

***Correspondence:**
Martin G. Frasch, Département d'Obstétrique-Gynécologie, Université de Montréal, CHU Sainte-Justine Centre de Recherche, 3175 Chemin de la Côte Ste-Catherine, Montréal, QC H3T 1C5, Canada
e-mail: mg.frasch@umontreal.ca

[†]Alex Xu and Lucien Daniel Durosier have contributed equally to this work.

Objective: Repetitive umbilical cord occlusions (UCOs) in ovine fetus leading to severe acidemia result in adaptive shut-down of electrocortical activity [electrocorticogram (ECoG)] as well as systemic and brain inflammation. We hypothesized that the fetuses with earlier ECoG shut-down as a neuroprotective mechanism in response to repetitive UCOs will show less brain inflammation and, moreover, that chronic hypoxia will impact this relationship.

Methods: Near-term fetal sheep were chronically instrumented with ECoG leads, vascular catheters, and a cord occluder and then underwent repetitive UCOs for up to 4 h or until fetal arterial pH was <7.00. Eight animals, hypoxic prior to the UCOs (SaO_2 <55%), were allowed to recover 24 h post insult, while 14 animals, 5 of whom also were chronically hypoxic, were allowed to recover 48 h post insult, after which brains were perfusion-fixed. Time of ECoG shut-down and corresponding pH were noted, as well as time to then reach pH <7.00 (ΔT). Microglia (MG) were counted as a measure of inflammation in gray matter layers 4–6 (GM4–6) where most ECoG activity is generated. Results are reported as mean ± SEM for $p < 0.05$.

Results: Repetitive UCOs resulted in worsening acidosis over 3–4 h with arterial pH decreasing to 6.97 ± 0.02 all UCO groups' animals, recovering to baseline by 24 h. ECoG shut-down occurred 52 ± 7 min before reaching pH <7.00 at pH 7.23 ± 0.02 across the animal groups. MG counts were inversely correlated to ΔT in 24 h recovery animals ($R = -0.84$), as expected. This was not the case in normoxic 48 h recovery animals, and, surprisingly, in hypoxic 48 h recovery animals, this relationship was reversed ($R = 0.90$).

Conclusion: Adaptive brain shut-down during labor-like worsening acidemia counteracts neuroinflammation in a hypoxia- and time-dependent manner.

Keywords: fetus, microglia, ECoG, EEG, hypoxia, acidemia, labor, sheep

INTRODUCTION

Human clinical studies with umbilical cord blood gas and pH assessment at birth indicate an increasing risk for neonatal adverse outcome and longer-term sequellae including cerebral palsy with pH values <7.00 (1–3). Additionally, growth-restricted infants with chronic hypoxemia due to placental dysfunction are at greater risk for concerning acidemia at birth and thereby subsequent adverse neurological outcomes due to superimposed acute hypoxemia during labor (4–7). This is supported by studies in the ovine fetus showing that pre-existing hypoxia alters cerebral and cardiovascular responses to labor-like umbilical cord occlusions (UCOs) (8). This has led to the use of electronic fetal heart rate (FHR) monitoring as the main stay for the assessment of fetal health during labor (1–3). The absence of FHR decelerations along with presence of FHR variability is highly predictive for normal fetal blood gas/pH at birth (1–3). However, clinical FHR monitoring has a low positive predictive value for concerning acidemia at birth (~50%), so there is continued need for improving existing technologies for the detection of fetal hypoxic-acidemia during labor. (1–3)

We recently studied patterns of electrocortical activity [electrocorticogram (ECoG)] and FHR in the near-term ovine fetus in response to repetitive UCOs insults as might be seen in human labor, to delineate the time-course and correlation of ECoG change with worsening acidemia (7). There were consistent changes in ECoG with amplitude suppression and frequency increase during FHR decelerations in association with pathological decreases in fetal arterial blood pressure (ABP). These changes in ECoG suggested an "adaptive brain shut-down" and occurred on average 50 min prior to attaining a severe degree of acidemia with fetal arterial pH <7.00. Translational implications are that fetal ECoG monitoring can improve the positive predictive value of FHR monitoring for worsening acidemia at birth. We have shown

that emergence of pathological decreases in ABP in response to UCO-triggered FHR decelerations is not required for the observed correlated ECoG–FHR changes. This suggests that these changes are induced neurally in the context of an adaptive brain shut-down (7). The relationship between these cerebral responses and acidemia-triggered brain inflammation remains unknown (7, 9). If neuroinflammation is diminished by adaptive brain shut-down, then expedited delivery during labor when observing this "ECoG–FHR shut-down pattern" may improve postnatal brain development by reducing the risk for sustained postnatal neuroinflammation.

Based on our previous findings for fetal ECoG (7, 10), we hypothesized that fetuses with an earlier adaptive brain shut-down will show less neuroinflammation due to the neuroprotective effect of decreasing cerebral metabolic rate. Consequently, in the present study we quantified neuroinflammation in response to worsening acidemia as microglial activation in the fetal brain at 24 and 48 h following the cessation of the UCOs.

MATERIALS AND METHODS

SURGICAL PREPARATION

Twenty two near-term ovine fetuses [124 ± 1 days gestational age (GA), term = 145 days] of mixed breed were surgically instrumented. The anesthetic and surgical procedures and post-operative care of the animals have been previously described (4, 5). Briefly, polyvinyl catheters were placed in the right and left brachiocephalic arteries and the right cephalic vein. Stainless steel electrodes were sewn onto the fetal chest to monitor the electrocardiogram (EKG). Stainless steel electrodes were additionally implanted biparietally on the dura for the recording of ECoG. An inflatable silicon rubber cuff (*In vivo* Metric, Healdsburg, CA, USA) for UCO induction was also placed around the proximal portion of the umbilical cord and secured to the abdominal skin. Once the fetus was returned to the uterus, a catheter was placed in the amniotic fluid cavity and another in the maternal femoral vein. Antibiotics were administered intravenously to the mother (0.2 g trimethoprim and 1.2 g sulfadoxine, Schering Canada Inc., Pointe-Claire, QC, Canada) and the fetus and into the amniotic cavity (1 million IU penicillin G sodium, Pharmaceutical Partners of Canada, Richmond Hill, ON, Canada). Amniotic fluid lost during surgery was replaced with warm saline. The uterus and abdominal wall incisions were sutured in layers and the catheters exteriorized through the maternal flank and secured to the back of the ewe in a plastic pouch.

Postoperatively, animals were allowed 4 days to recover prior to experimentation and daily antibiotic administration was continued. Arterial blood was sampled for evaluation of fetal condition and catheters were flushed with heparinized saline to maintain patency. Animals were 128 ± 1 days GA on the first day of experimental study. Animal care followed the guidelines of the Canadian Council on Animal Care and was approved by the University of Western Ontario Council on Animal Care.

EXPERIMENTAL PROCEDURE

The animals were studied over a ~6 h period in three groups. Fetal chronic hypoxia was defined as arterial O_2Sat <55% as measured on post-operative days 1–3 and at baseline prior to beginning the UCOs. The first group comprised eight animals spontaneously hypoxic that were subjected to repetitive UCOs followed by 24 h recovery with subsequent necropsy to assess neuroinflammation (H/UCO 24). While these ECoG and neuroinflammation results have been published (7, 11), they were not interpreted together and are included here to address the new question of the relationship between the degree of adaptive brain shut-down and neuroinflammation. The second group comprised five fetuses that were also spontaneously hypoxic but studied at 48 h recovery (H/UCO 48). The third group of fetuses was normoxic (O_2Sat >55% before UCOs) and formed an N/UCO 48 h recovery group ($n = 9$, N/UCO 48). That is, the second and third UCO groups were allowed to recover for 48 h after the UCOs with subsequent necropsy to assess neuroinflammation.

The protocol for the first group has been reported (7, 11) and is essentially similar in the objective to the protocol described below in that it mimics uterine contractions during human labor. The protocol of the H/UCO 48 and N/UCO 48 groups has also been reported (12, 13). The difference between the groups with regard to mimicking the uterine contractions during labor is that in the H/UCO group with 24 h recovery time, the UCOs' frequency was increased with each UCO being a complete one, while in the H/UCO and N/UCO groups with 48 h recovery the frequency was kept constant and the completeness of the UCO series was increased progressively. The end result was the same: a cumulative, worsening acidemia. Hence, we believe that this difference in experimental design has no confounding impact on our findings. After a 1–2 h baseline control period, the second and third groups of animals underwent mild, moderate, and severe series of repetitive UCOs by graduated inflation of the occluder cuff with a saline solution. During the first hour following the baseline period, mild variable FHR decelerations were performed with a partial UCO for 1 min duration every 2.5 min, with the goal of decreasing FHR by ~30 bpm, corresponding to an ~50% reduction in umbilical blood flow (14, 15). During the second hour, moderate variable FHR decelerations were performed with increased partial UCO for 1 min duration every 2.5 min with the goal of decreasing FHR by ~60 bpm, corresponding to an ~75% reduction in umbilical blood flow (15). Animals then underwent severe variable FHR decelerations with complete UCO for 1 min duration every 2.5 min until the targeted fetal arterial pH of <7.0 was detected or 2 h of severe UCO had been carried out, at which point the repetitive UCOs were terminated. These animals were then allowed to recover for 48 h following the last UCO. Fetal arterial blood samples were drawn at baseline, at the end of the first UCO of each series (mild, moderate, severe), and at 20 min intervals (between UCOs) throughout each of the series, as well as at 1, 24, and 48 h of recovery. For each UCO series, blood gas sample and the 24 h recovery sample, 0.7 mL of fetal blood was withdrawn, while 4 mL of fetal blood was withdrawn at baseline, at pH nadir <7.00, and at 1 and 48 h of recovery. The amounts of blood withdrawn were documented for each fetus and replaced with an equivalent volume of maternal blood at the end of day 1 of study.

All blood samples were analyzed for blood gas values, pH, glucose, and lactate with an ABL-725 blood gas analyzer (Radiometer Medical, Copenhagen, Denmark) with temperature corrected to

39.0°C. Plasma from the 4 mL blood samples was frozen and stored for cytokine analysis, and will be reported separately.

After the 24 or 48 h recovery blood sample, the ewe and the fetus were killed by an overdose of barbiturate (30 mg sodium pentobarbital IV, MTC Pharmaceuticals, Cambridge, ON, Canada). A post mortem was carried out during which fetal sex and weight was determined and the location and function of the umbilical occluder were confirmed. The fetal brain was perfusion-fixed and subsequently dissected and processed for later immunohistochemical study as previously reported (11).

DATA ACQUISITION AND ANALYSIS

A computerized data acquisition system was used to record fetal arterial and amniotic pressures, the ECG and ECoG electrical signals, as previously described (16), which were monitored continuously throughout the baseline, UCO series, and first hour of the recovery period (**Figure 1**). Arterial and amniotic pressures were measured using Statham pressure transducers (P23 ID; Gould Inc., Oxnard, CA, USA). ABP was determined as the difference between instantaneous values of arterial and amniotic pressures. A PowerLab system was used for data acquisition and analysis (Chart 7 For Windows, ADInstruments Pty Ltd., Castle Hill, NSW, Australia).

Pressures, ECG, and ECoG were recorded and digitized at 1000 Hz for further study. For ECG, a 60 Hz notch filter was applied, while for ECoG, a band pass 0.3–30 Hz filter was used. FHR was triggered and calculated online from arterial pressure systolic peaks.

Averaged values of FHR and ABP were calculated from artifact-free recordings of 1 h of baseline, as well as between and during each consecutive variable FHR deceleration induced by the mild, moderate, and severe UCOs as previously reported (12).

Electrocorticogram was sampled down to 100 Hz prior to the analysis. For each animal, mean values of the ECoG amplitudes were determined at baseline as well as during and between UCO for each of the UCO deceleration series.

Immunohistochemistry

The presence of microglia (MG) in brain tissue sections was determined by avidin–biotin-peroxidase complex enhanced immunohistochemistry (Vectastain Elite; Vector Laboratories, Burlingame, CA, USA) as previously reported (9, 11). To reduce staining

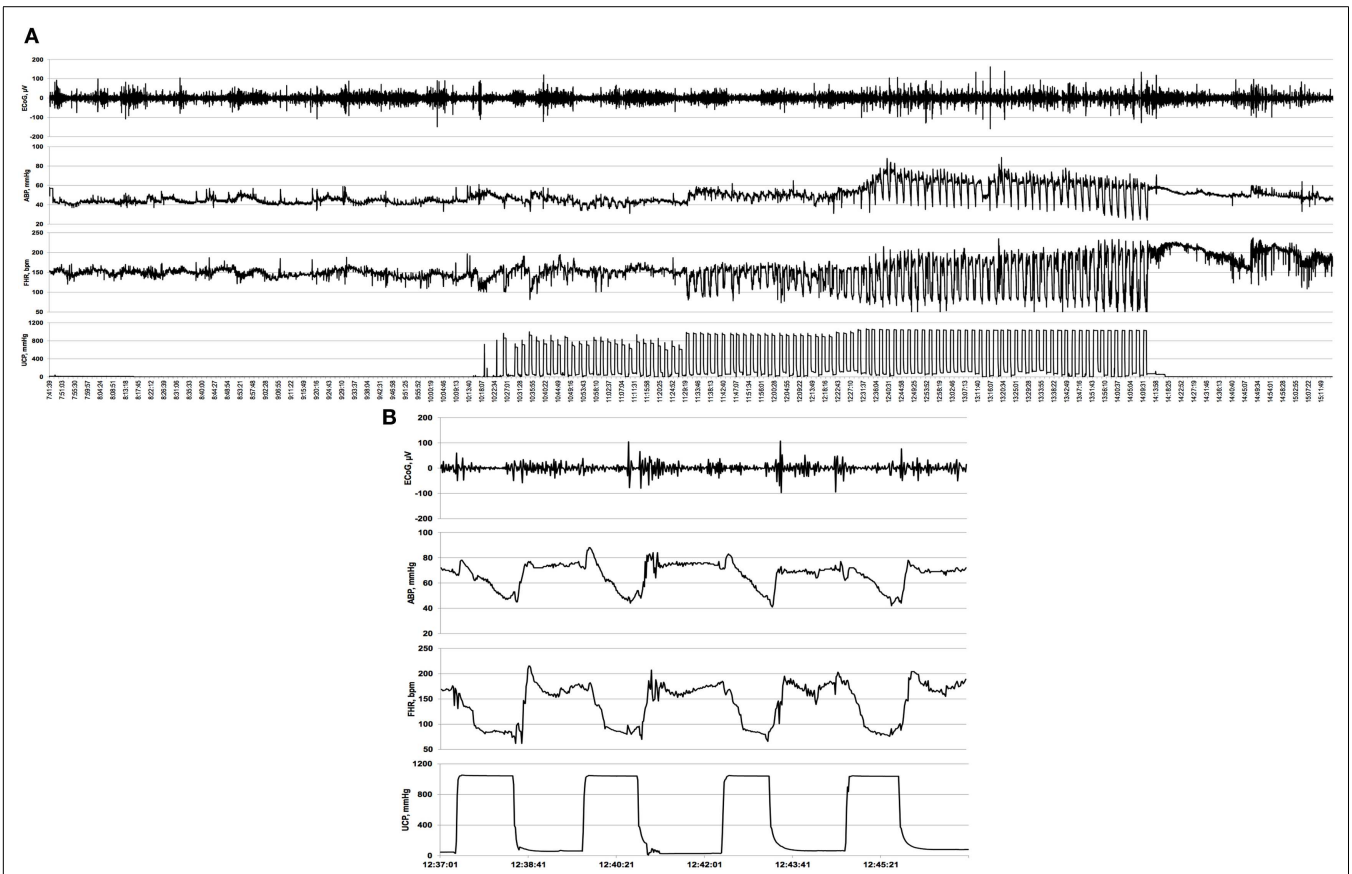

FIGURE 1 | Example of an individual electrocorticogram (ECoG) response to repetitive umbilical cord occlusions (UCOs). **(A)** A complete 5 h recording is shown with baseline, mild, moderate, and severe UCOs. UCOs occurrences are indicated by the UCP channel: umbilical contraction pressure increases correspond to an occlusion of the umbilical cord. Note emergence of the adaptive brain shut-down pattern visible in ECoG- and fetal heart rate (FHR) synchronization and the accompanying changes in arterial blood pressure (ABP). **(B)** Ten minutes zoomed-in window of this synchronized ECoG–FHR pattern.

variability, all immunohistochemistry was performed on the same day with the same batch of antibody and solutions. Tissue sections were incubated with an anti-IBA1 rabbit polyclonal antibody (1:500, Wako Industries, Richmond, VA, USA), a robust marker for sheep MG, with detection of bound antibody obtained following incubation in Cardassian DAB Chromogen (Biocare Medical, Concord, CA, USA).

Brain regions that were selected from each animal for analysis were taken from a coronal section of blocked cerebral hemisphere tissue at the level of the mammillary bodies and included the parasagittal and convexity cerebral gray matter, periventricular white matter, thalamus, CA1, dentate gyrus (DG), and the combined CA2 and CA3 regions of the hippocampus. Each of the gray matter regions was further divided into sub-regions combining layers 1, 2, and 3 and layers 4, 5, and 6. Image analysis was performed with a transmitted light microscope (Leica DMRB, Leica-Microsystems, Wetzler, Germany) at 40× magnification. Positive MG cell immunostaining was quantified with an image analysis program (Image Pro Plus 6.0, Media Cybernetics, Silver Spring, MD, USA). The image analysis system was first calibrated for the magnification settings that were used, and thresholds were established to provide even lighting and no background signal. Six high-power field (HPF) photomicrographs (HPF area = 7 cm^2) per brain region/subregion per animal were collected as a 24 bit RGB color modeled image. The same illumination setting was applied to all images for all of the brain regions. Using the Image Pro Plus' RGB color range selection tool, color samplings of positive DAB stained areas were obtained from multiple brain regions and tested for specificity against the negative control. Appropriate ranges of color were selected showing positive contiguous cytoplasmic staining as a criterion for MG cell count scoring, which were then applied uniformly to calibrated images for all brain regions. Scoring was performed in a blinded fashion to the N/UCO, H/UCO, and control (no UCO) groups. Specifically, to perform the MG counts normalization procedure for inter-group comparisons outlined below, the respective control groups were analyzed as follows: a hypoxic control group (i.e., no UCOs, same recovery time, $N = 5$) matched to H/UCO 24, another normoxic control group matched to N/UCO 48 (no UCOs, same recovery time, $N = 10$), and a hypoxic control group (i.e., no UCOs, $N = 4$). In the current study, our focus is on the neuroinflammation in the gray matter layers 4–6 (GM4–6) where most ECoG activity is generated. Neuroinflammation results in other brain regions for H/UCO 24 have been reported (11) and for H/UCO 48 and N/UCO 48 will be reported separately.

Normalization of MG counts to allow comparison between the 24 and 48 h recovery groups

Different observers scored H/UCO 24 and H/UCO 48 and N/UCO 48 groups. This is due to the fact that the respective research projects took place at different time points. This necessitated a normalization of each group's MG counts to eliminate any observer-dependent variability in MG scoring thus allowing an inter-group comparison of MG counts. To achieve that goal, we calculated average values of MG counts in each brain region for the control animals in each of the three groups. The values were then used to normalize the individual regional MG counts in each of the intervention groups, thus allowing an inter-group comparison.

Analysis of the relation between neuroinflammation and ECoG activity

Data from the first group of animals (7, 11) were pooled together with the current findings in the second and third groups. As noted, in the first group, eight animals, all hypoxic prior to the UCOs, were allowed to recover 24 h post insult, while the 14 animals currently studied formed the cohort that was allowed to recover 48 h post insult, after which brains were perfusion-fixed. The individual time of fetal adaptive brain shut-down onset was noted as observed at the start of the synchronized ECoG/FHR changes. The difference between this time and the time to then reach the target pH <7.00 was calculated as ΔT. The normalized MG counts in the GM4–6 were then correlated to ΔT.

STATISTICAL ANALYSIS

Normal data distribution was tested using Kolmogorov–Smirnov test followed by parametric or non-parametric tests, as appropriate. Arterial pH, lactate, and base excess (BE) measurements in response to repetitive UCOs at the onset the adaptive brain shut-down and at pH nadir were compared with the corresponding baseline values by one-way repeated-measures analysis of variance (ANOVA) with Student–Newman–Keuls *post hoc* analysis. One-way repeated-measures ANOVA followed by Holm–Sidak (versus baseline) or Student–Newman–Keuls tests for multiple comparisons have been used to assess differences in ECoG and cardiovascular responses to UCOs within each UCO group. Differences in ECoG and cardiovascular alterations during the synchronized ECoG–FHR pattern were tested using *t*-test or signed rank test (i.e., pairwise during versus between UCO during adaptive brain shut-down).

Between group differences of the values of the fetal body and brain weights as well as brain/body ratios were assessed using ANOVA or Kruskal–Wallis ANOVA on ranks with Holm–Sidak or Dunn's tests for multiple pairwise comparisons, respectively. Between group differences of the values of the arterial O_2Sat, the ECoG and cardiovascular variables at each time point and the normalized MG counts were assessed using rank-sum test. No adjustment for multiple comparisons was undertaken at this point (17).

Spearman correlation analysis was performed and *R* values are presented where $p < 0.05$ (SPSS 19; IBM, Armonk, NY, USA). All values are expressed as means ± SEM. Statistical significance was assumed for $p < 0.05$.

RESULTS

GENERAL CHARACTERISTICS OF THE EXPERIMENTAL GROUPS

Fetal acid–base status, cardiovascular parameters FHR and ABP as well as ECoG amplitudes were within physiological range in all groups except for a lower baseline ECoG amplitude in H/UCO 24 compared to the N/UCO 48 (**Tables 1** and **2**). In the H/UCO 24, arterial O_2Sat measured 50 ± 3% ($p = 0.001$ versus N/UCO 48 and $p = 0.27$ versus H/UCO 48). In the N/UCO 48, arterial O_2Sat measured 65 ± 2%, which was higher than in the H/UCO 48 where it measured 41 ± 6% ($p < 0.05$). Fetal body weights at 2.8 ± 0.2 kg

Table 1 | Acid–base status.

	Baseline	Pattern	pH nadir
H/UCO 24 h GROUP			
pH	7.36 ± 0.10	7.24 ± 0.04*	6.90 ± 0.04*
Base excess (mmol/L)	3.7 ± 0.5#	−3.5 ± 2.1*	−16.6 ± 1.0*
Lactate (mmol/L)	1.6 ± 0.2	6.3 ± 1.5*	15.6 ± 0.3*,#
H/UCO 48 h GROUP			
pH	7.34 ± 0.01	7.23 ± 0.01*	7.01 ± 0.03*
Base excess (mmol/L)	2.0 ± 0.7	−2.8 ± 1.6*	−15.5 ± 0.3*
Lactate (mmol/L)	2.5 ± 0.9	4.8 ± 0.8*	11.9 ± 3.1*
N/UCO 48 h GROUP			
pH	7.35 ± 0.01	7.17 ± 0.03*	7.00 ± 0.03*
Base excess (mmol/L)	1.6 ± 0.7	−6.4 ± 1.3*	−13.6 ± 1.1*
Lactate (mmol/L)	2.0 ± 0.5	5.7 ± 1.1*	10.3 ± 1.7*

Values are shown during baseline (prior to UCOs), between the UCOs at the onset of the synchronized ECoG–FHR pattern and at pH nadir (pH <7.00) for the hypoxic 24 h recovery group (H/UCO, n = 8), the normoxic 48 h recovery groups (N/UCO, n = 9) as well as the hypoxic 48 h recovery group (H/UCO, n = 5). Values are presented as mean ± SEM.

*Within group: *p < 0.05 versus baseline.*

Between groups: #p < 0.05 for H/UCO 24 h recovery group versus N/UCO 48 h recovery group. We found no differences between the groups at the time of the synchronized ECoG–FHR pattern onset.

Table 2 | Brain electrical and cardiovascular responses to umbilical cord occlusions (UCOs).

	Baseline	Pattern	
		dur UCO	btw UCO
H/UCO 24 h GROUP			
ΔT (min)			52 ± 13
ECoG amplitude (μV)	66 ± 12[c]	54 ± 9[b,c]	106 ± 21[a,c]
FHR (bpm)	162 ± 13	91 ± 12[a,b]	153 ± 12
ABP (mmHg)	42 ± 2	50 ± 3[a,b]	57 ± 4[a]
H/UCO 48 h GROUP			
ΔT (min)			59 ± 15
ECoG amplitude (μV)	100 ± 13	76 ± 10[b]	173 ± 27[a]
FHR (bpm)	168 ± 5	85 ± 7[a]	112 ± 10[c]
ABP (mmHg)	48 ± 3	60 ± 6	63 ± 6
N/UCO 48 h GROUP			
ΔT (min)			48 ± 12
ECoG amplitude (μV)	127 ± 14	102 ± 17[b]	209 ± 26[a]
FHR (bpm)	159 ± 5	101 ± 6[a,b]	171 ± 8
ABP (mmHg)	44 ± 2	57 ± 2[a]	60 ± 2[a]

Fetal heart rate (FHR, beats per minute, bpm), arterial blood pressure (ABP), and electrocorticogram (ECoG) amplitude values are shown at baseline and during the synchronized ECoG–FHR pattern (during versus between UCOs) for the hypoxic 24 h recovery group (H/UCO, n = 8), the normoxic 48 h recovery groups (N/UCO, n = 9) as well as the hypoxic 48 h recovery group (H/UCO, n = 5). ΔT is calculated as the difference between the individual times of fetal adaptive brain shut-down onset (observed at the start of the synchronized ECoG/FHR change) and the time to then reach the target pH <7.00. Values are presented as mean ± SEM.

Within group:

[a] versus baseline;

[b] during versus between UCO (p < 0.01).

Between groups:

[c] p < 0.05, H/UCO 24 h recovery or H/UCO 48 h recovery versus N/UCO 48 h recovery group.

in the H/UCO 24 group were lower than in both H/UCO 48 and N/UCO 48 groups at 3.7 ± 0.2 and 4.1 ± 0.2 kg, respectively (both $p < 0.01$). Fetal brain weights at 40 ± 1 g in the H/UCO 24 group were lower than in the N/UCO 48 group at 45 ± 1 g ($p < 0.01$) and not different from the values in the H/UCO 48 group at 41 ± 0.2 g. Consequently, the brain/body weight ratio at 15 ± 1 was higher in the H/UCO 24 than in N/UCO 48 group (11 ± 0.6, $p < 0.05$), but not different from that in the H/UCO 48 group at (11 ± 0.4). Across all groups, higher body and brain weights correlated with larger ECoG amplitude at baseline ($R = 0.60$ and $R = 0.73$, respectively; both $p < 0.01$). This correlation was found in the H/UCO 24 and N/UCO 48 groups with $R = 0.73$ ($p = 0.02$) and $R = 0.70$ ($p = 0.05$), respectively, but not in the H/UCO 48 group ($R = 0.21$, $p = 0.73$, **Figure 2**).

BRAIN AND CARDIOVASCULAR RESPONSES TO THE REPETITIVE UMBILICAL CORD OCCLUSIONS

Repetitive UCOs resulted in worsening acidosis over 3–4 h with arterial pH decreasing from an average pH of 7.35 ± 0.01 at baseline to an average of 6.97 ± 0.02 across the groups and with all animals recovering to baseline by 24 h (**Table 1**) (12, 18). The average baseline lactate and BE were 1.9 ± 0.3 and 2.6 ± 0.4 mmol/L, respectively, increasing/decreasing to an average of 12.6 ± 1.1 and −15.1 ± 0.6 mmol/L at pH nadir, respectively (both $p < 0.01$, **Table 1**). Consistent with the chronic hypoxic status, in the N/UCO 48 h recovery group, baseline BE was ~1/2 that of the H/UCO 24; similarly, at pH nadir, lactate was ~2/3 that of the H/UCO 24 (both $p < 0.05$, **Table 1**).

Cardiovascular and ECoG responses to the UCOs have been presented elsewhere (7, 12, 18). Here, we focus on the effect of adaptive brain shut-down represented by the emergence of the ECoG–FHR synchronization as it may pertain to the neuroinflammation.

Electrocorticogram adaptive shut-down occurred 52 ± 7 min (28–69 min, 25th–75th quartiles) before reaching pH <7.00 at pH 7.23 ± 0.02 across the animal groups with no difference of ΔT between the groups (**Table 2**, **Figure 1**). During the adaptive shut-down, ECoG amplitude began to consistently decrease to 79 ± 9 from 164 ± 17 μV on average during versus between each UCO, respectively ($p < 0.001$). Notably, we observed no differences in pH, lactate, or BE between the groups at the time of the onset of the ECoG–FHR synchronization. The average values of arterial lactate and BE at this time were 4.8 ± 0.8 and −3.4 ± 1.6 mmol/L, respectively. These values were obtained from the most proximal blood sample taken at 20 min intervals during the experiment, i.e., usually within ~10 min from the ECoG–FHR synchronization onset.

EFFECTS OF PRECEDING HYPOXIA ON CARDIOVASCULAR RESPONSES AND ECoG

We found no effect of chronic hypoxia on pH, lactate, or BE at any time point (baseline, emergence of ECoG–FHR synchronization or

pH nadir) nor on baseline ECoG amplitude (**Table 2**). Moreover, chronic hypoxia preceding UCOs of increasing severity had no impact on the average timing of ~53 min prior to pH drop to <7.00 when adaptive brain shut-down was observed. There was, however, a pronounced impact of preceding hypoxia on cardiovascular and ECoG responses to the UCOs. During the ECoG–FHR synchronized activity, H/UCO 48 fetuses showed a ~35% lower mean FHR between UCOs than their normoxic counterparts (N/UCO 48) ($p < 0.01$, **Table 2**). During and between the UCOs, when the ECoG–FHR synchronized activity was observed, ECoG amplitude was 50% lower in the H/UCO 24 than in the N/UCO 48 ($p < 0.05$ and $p < 0.01$, respectively).

ECoG–FHR SYNCHRONIZATION MODULATES NEUROINFLAMMATION AND INJURY

To test the hypothesis that neuroinflammation is diminished by adaptive brain shut-down, we studied MG counts in the GM4–6, the brain region where most ECoG activity is generated (**Figure 3**). Within each group, there were no differences between the MG counts in the GM4–6 region of UCO group animals compared to the respective controls. Normalized GM4–6 MG counts were higher in H/UCO 24 animals than H/UCO 48 and N/UCO 48 animals (both $p < 0.05$; **Figure 4**). In contrast, MG counts in the N/UCO 48 animals were not different from those in the H/UCO 48 animals. Supporting our hypothesis, MG counts were inversely correlated to ΔT in the H/UCO 24 animals ($R = -0.84$), but not in N/UCO 48 animals, and surprisingly in H/UCO 48 animals this relationship was reversed ($R = 0.90$, **Figure 4**).

DISCUSSION

The chief findings of this study are that higher MG counts in GM4–6 of the H/UCO 24 compared to both H/UCO 48 and N/UCO 48 animals indicate a rapid onset/offset of the fetal brain inflammatory response with repetitive UCOs and severe acidemia, while the inverse correlation to ΔT supports the earlier onset of ECoG shut-down as an important neuroprotective mechanism limiting MG activation at 24 h post UCO insult. In contrast, albeit at a lower level compared to the H/UCO 24, we found relatively higher MG counts in the H/UCO 48 in those fetuses that showed an earlier adaptive brain shut-down. How can we explain this time-specific effect of the shut-down of neuronal metabolism on neuroinflammation?

DOES FETAL CHRONIC HYPOXIA ALTER CEREBRAL RESPONSE TO AN ACUTE HYPOXIC-ACIDEMIA INSULT?

It is possible that chronic hypoxia primes fetal brain re-exposure to an inflammatory stimulus such as acidemia, which then is potentiated or induced by (further) lactate accumulation during an adaptive brain shut-down triggered by worsening acidemia. This may be due to several inter-related reasons. First, fetal brains may be less capable of metabolizing the lactate primarily due to pre-existing hypoxia with curbed metabolism and lesser ATP reserves to sustain the anti-inflammatory adenosine mono-phosphate kinase (AMPK) activation status beyond an acute response (19–22). Second, altered cardiovascular responses to UCOs in the H/UCO group fetuses with resulting stress on the auto-regulatory capacity of the cerebral blood flow may further contribute to the gradual exhaustion of the cerebral metabolic reserves in these fetuses (23, 24). Third, hypoxic fetal brains that shut-down earlier may accumulate at 48 h post UCOs a relatively higher level of regional lactate and other inflammatory mediators.

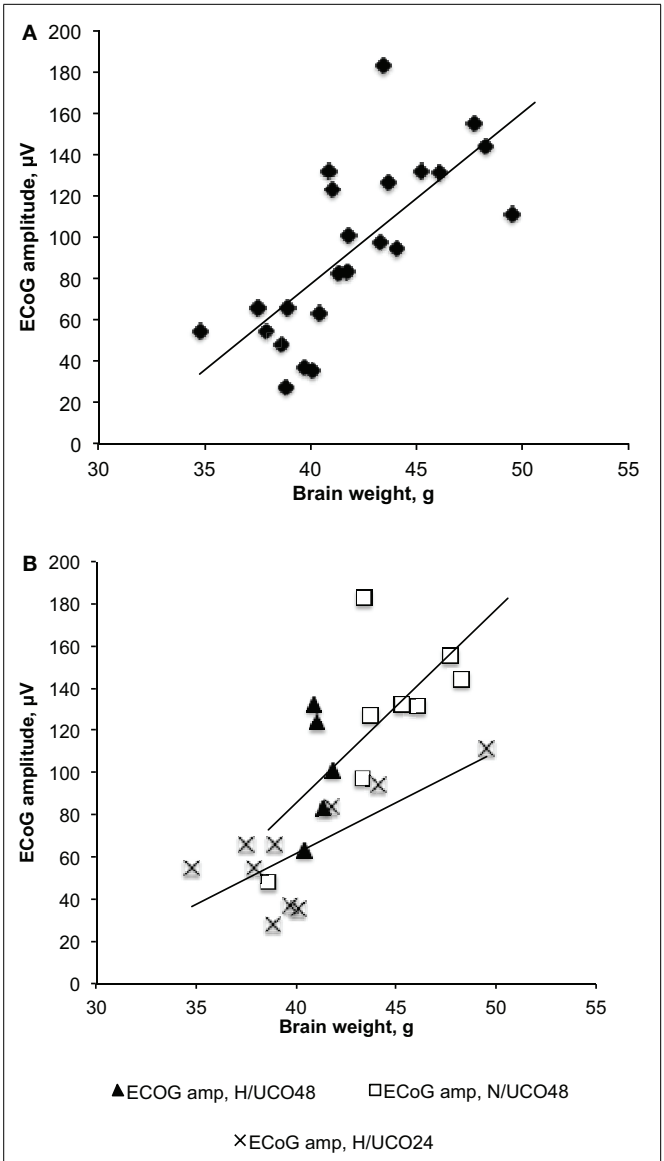

FIGURE 2 | Fetal brain weights' correlations to the amplitudes of electrocorticogram (ECoG) at baseline, i.e., prior to commencing with umbilical cord occlusions. **(A)** Correlation across all three groups showing increasing fetal ECoG amplitude with increasing brain weight. **(B)** Group-specific correlations still hold true despite lower brain weights in the H/UCO 24 h group compared to the N/UCO 48 h group.

Rising cerebral extracellular acidosis reduces EEG seizure activity via activation of inhibitory interneurons by acid-sensing ion channels 1a (ASIC1a) (25). Thus, even extracellular brain acidosis *without* hypoxia increases neuronal inhibitory tone. This lends support to our hypothesis that the observed cyclical changes in amplitude and frequency of the fetal ECoG during the ECoG–FHR synchronization at arterial pH ~7.23 and lower are due to unmasking of inhibitory interneuron activity during the UCOs

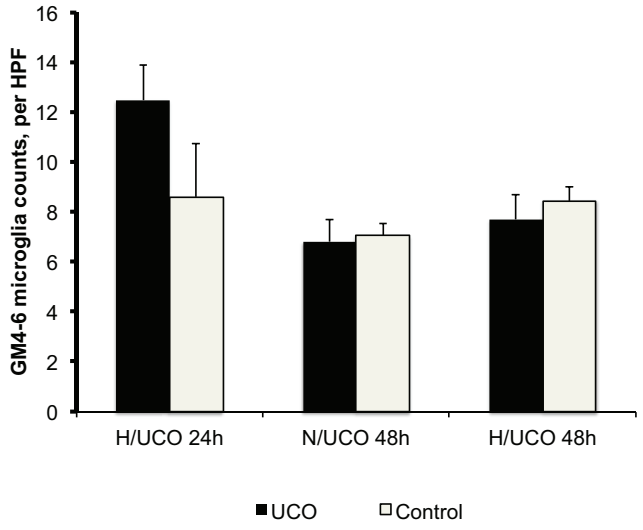

FIGURE 3 | Neuroinflammation in gray matter layers 4–6 (GM4–6) assessed as microglia (MG) counts per high-power field (HPF) at 24 and 48 h post insult in normoxic (N/UCO) and hypoxic (H/UCO) groups versus respective control groups. Cf. **Figure 3** for the inter-group comparison. H/UCO 24 data reproduced with kind permission from Elsevier.

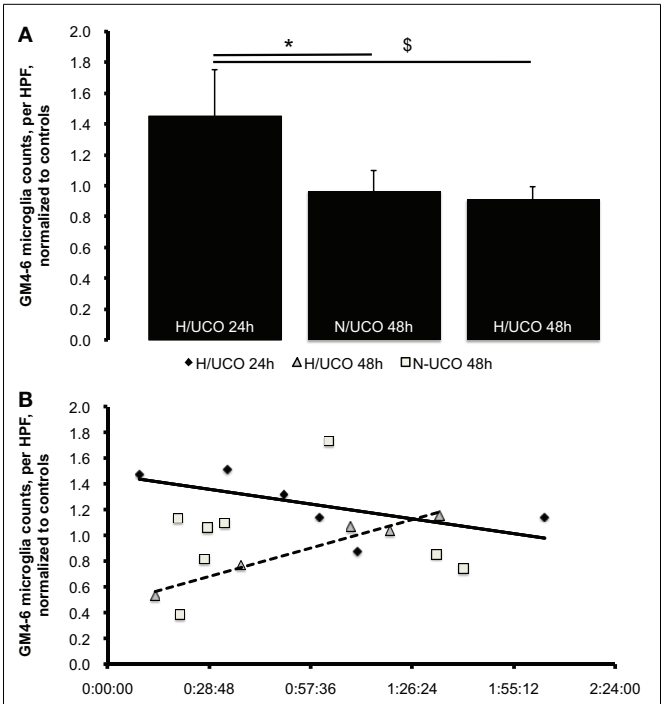

FIGURE 4 | (A) Neuroinflammation in gray matter layers 4–6 (GM4–6) assessed as microglia (MG) counts per high-power field (HPF) at 24 and 48 h post insult, normalized by average MG counts per HPF in respective control groups' brain regions (to allow for inter-group comparison, see "Materials and Methods" for details). Mean ± SEM. $^{*}p = 0.03$ for N/UCO 48 h versus H/UCO 24 h group; $^{\$}p = 0.02$ for H/UCO 48 h versus H/UCO 24 h group. **(B)** Correlation to adaptive brain shut-down timing expressed by ΔT as the difference between the individual times of fetal adaptive brain shut-down onset (observed at the start of the synchronized ECoG/FHR change) and the time to then reach the target pH <7.00. Spearman correlation coefficients $R = -0.84$ for H/UCO 24 h group and $R = 0.90$ for H/UCO 48 h group (both $p = 0.04$); no significant correlation for N/UCO 48 h group ($R = -0.05$, $p = 0.91$). Due to artifacts in ECoG, ΔT was missing in two out of eight H/UCO 24 h group animals and in one animal from the N/UCO 48 h group.

(7). Metabolic, respiratory, or mixed acidemia with pH <7.25, also in absence of hypoxia, results in reversible EEG changes in preterm neonates of <32 weeks with increasing inter-burst intervals indicating a global alteration of cerebral blood flow and related shut-down of brain electrical activity (26). Other mechanisms may also be involved in the observed ECoG dynamics and ensuing neuroinflammation. They may include depolarization block and adenosine (A1) receptor-mediated decrease in neuronal activity. (27, 28) It remains to be explored whether these processes can explain the temporal profile of the ECoG changes we have observed.

The requirement of acidemia to produce neuroinflammation is supported by the findings in fetal sheep near term that chronic hypoxia does not lead to microglial activation and acidemia is required to induce neuroinflammation (9, 29). Moreover, isolated systemic hypoxia without ischemia does not cause neuronal necrosis as shown in middle cerebral artery occlusion model of stroke in adult rats, however, it will exacerbate ischemic necrosis (30). In line with these findings, pediatric patients <9 months old with proven hypoxic or hypotensive episodes due to perinatal asphyxia, congenital heart defects, or chronic pulmonary dysfunction were found to often show a dense infiltrate of microglial cells in the dendate gyrus making this neuroinflammation a neuropathological marker of mild hypoxic–ischemic brain injury (31). That is, neuroinflammation, preceding brain necrosis, plays a pivotal role in mediating brain injury induced by neonatal hypoxic–ischemic encephalopathy, the neuropathological substrate of cerebral palsy (32). Meanwhile, suspending neuronal activity is known to result in prolonged preservation of viability in adults and neonates (33, 34). This makes our present observations plausible that neuroinflammation is mitigated by an early adaptive neuronal shut-down in response to repetitive intermittent hypoxic episodes with cumulative acidemia.

METHODOLOGICAL CONSIDERATIONS

As outlined in Section "Materials and Methods," we believe that the difference in experimental design had no confounding impact on our findings, since both approaches produced a worsening acidemia within a similar time frame. This is also supported by the fact that all groups had normal pH values at baseline and that the adaptive brain shut-down was uniformly observed around the same time and around the same pH, lactate, and BE values. Moreover, all groups recovered their blood gas and acid–base status to baseline within 24 h as reported (7, 12, 18). However, the chronic hypoxia in the animals of the H/UCO 24 was accompanied by a higher BE at baseline and reaching a higher lactate at pH nadir than in the N/UCO 48. Moreover, the correlation between the timing of onset of adaptive brain shut-down and neuroinflammation was not observed in the N/UCO 48, but only in both chronically

hypoxic UCO groups. Thus, we propose that any impact of the experiments on the neuroinflammation measured at 24 or 48 h of recovery is due to the presence or absence of preceding spontaneous chronic hypoxia. The ECoG amplitude difference between the H/UCO 24 and N/UCO 48 groups is likely attributable to differences in the animal size as we report herein. While the expected relationship between brain weight and ECoG amplitude held true in both groups, the generally lower brain weight in the H/UCO 24 group explains the lower ECoG amplitude in this group compared to N/UCO 48 group. This finding is further supported by the evidence of the asymmetric IUGR in the H/UCO 24 group, at least in comparison to the N/UCO 48 group. IUGR, especially an asymmetric IUGR, has been associated with impaired synaptogenesis and altered ECoG activity (35–37). Lower ECoG amplitude in conjunction with the above indicators of altered growth trajectory supports the notion that the H/UCO 24 group was different from N/UCO 48 group in the intended sense of being chronically hypoxic.

A weakness of the present study is that we could not evaluate the effect of UCOs on neuroinflammation at 24 h in fetuses that were normoxic to match the findings at 48 h in the N/UCO 48. Consequently, our data currently suggest that earliness of the onset of the adaptive brain shut-down confers no neuroprotection in the sense of reduced neuroinflammation to the fetuses that were normoxic prior to UCOs. Next, while adaptive brain shut-down represents a global brain response, most ECoG activity is generated in the cortical gray matter layers 4–6. Hence, here we reasoned that our primary focus in connecting ECoG to neuroinflammation should be on the microglial activity in these cortical layers. However, upper cortical layers and subcortical afferents also contribute to ECoG, albeit likely on a subtler level of ECoG properties that is not captured by the visually discernable ECoG changes characteristic of the adaptive brain shut-down. Hence, further studies are needed to delineate this relationship further and under varying conditions leading up to an adaptive brain shut-down.

CONCLUSION AND SIGNIFICANCE

The translational significance of our findings is that EEG–FHR monitoring during labor may not only identify fetuses at risk of developing severe acidemia ~1 h ahead of the pH drop to <7.00; EEG–FHR monitoring intrapartum may also provide information to the neonatologist as to which babies may be at higher risk of brain injury resulting in conditions such as cerebral palsy due to developing neuroinflammation. FHR monitoring may guide this diagnostic process by helping to distinguish chronically hypoxic fetuses prior to labor onset from the normoxic fetuses. The therapeutic implication is that neuroprotective drug treatments need to target the postnatal 24 h time window to benefit from the anti-neuroinflammatory effect the adaptive brain shut-down may have in the chronically hypoxic fetuses when they are born.

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What are the best animal models for testing early intervention in cerebral palsy?

Gavin John Clowry*, Reem Basuodan and Felix Chan

Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK

Edited by:
Christopher Mario Inglese, Loyola University Stritch College of Medicine, USA

Reviewed by:
Christopher Mario Inglese, Loyola University Stritch College of Medicine, USA
Nikolas Mata-Machado, Loyola University of Chicago, USA

****Correspondence:***
Gavin John Clowry, Institute of Neuroscience, Framlington Place, Newcastle University, Newcastle upon Tyne NE2 4HH, UK
e-mail: gavin.clowry@ncl.ac.uk

Interventions to treat cerebral palsy should be initiated as soon as possible in order to restore the nervous system to the correct developmental trajectory. One drawback to this approach is that interventions have to undergo exceptionally rigorous assessment for both safety and efficacy prior to use in infants. Part of this process should involve research using animals but how good are our animal models? Part of the problem is that cerebral palsy is an umbrella term that covers a number of conditions. There are also many causal pathways to cerebral palsy, such as periventricular white matter injury in premature babies, perinatal infarcts of the middle cerebral artery, or generalized anoxia at the time of birth, indeed multiple causes, including intra-uterine infection or a genetic predisposition to infarction, may need to interact to produce a clinically significant injury. In this review, we consider which animal models best reproduce certain aspects of the condition, and the extent to which the multifactorial nature of cerebral palsy has been modeled. The degree to which the corticospinal system of various animal models human corticospinal system function and development is also explored. Where attempts have already been made to test early intervention in animal models, the outcomes are evaluated in light of the suitability of the model.

Keywords: cerebral palsy, corticospinal tract, hypoxia/ischemia, perinatal stroke, periventricular white matter injury

INTRODUCTION

It is widely accepted that research with animal models is crucial to developing and testing new therapies. We need to understand the cellular mechanisms that underlie the organism's response to brain injury in the short and long term, and it is assumed that at the cellular level all mammals share these responses. However, there are drawbacks to this approach. It is important not to fall into the traps identified in pre-clinical adult stroke research, which may explain the massive failure rate in clinical trials of novel neuroprotective agents identified in animal experiments (1). These include omission of fundamental aspects of experimental design such as blinding, randomization, exclusion reporting, and sample size, but also "cherry picking" the data to publish to maximize impact (2). But it also seems to us that not enough time is spent asking how directly applicable to humans are our models?

Careful consideration has to be given as to the extent the animal model reflects human in terms of the way the nervous system functions and develops. Timing of experiments is crucial; for instance one of the significant drawbacks with studying rodents is the rapidity with which the CNS develops over days, compared to months in primate species, whereas, cellular processes of neuroinflammation are likely to occur on a more similar timescale between species. In this article, we ask what exactly are we trying to model? How similar are our animal models to the human condition? What have our animal models told us so far, and what outcomes should we be measuring in order to gage the likely success of our proposed therapies?

WHAT ARE WE TRYING TO MODEL?

CEREBRAL PALSY IN HUMANS

The incidence of cerebral palsy in the developed world is high, around 2 per 1000 live births or more (3). It is therefore a common condition that causes disability throughout life, which is often severe. Cerebral palsy is an umbrella term for a number of conditions including cerebellar ataxia and basal ganglia disorders, but this article will largely concentrate on the most common condition, spastic cerebral palsy (80% of cases) primarily arising from insults to the cerebral cortex and associated, sub-cortical white matter (4). Causal pathways are many and may interact with each other, indeed multiple causes, including a genetic predisposition to infarction, may need to interact to produce a clinically significant injury (4–6). The most commonly encountered causes are summarized in **Figure 1** and include periventricular white matter injury (PVWMI) in premature babies, which results from hypoxia/ischemia (H/I) in the periventricular regions around the lateral ventricles. This results, primarily, in damage to the subplate and developing sub-cortical axon tracts of the intermediate zone whilst the overlying gray matter is relatively spared. It generally causes spastic diplegia. In all, bilateral spasticity has a prevalence of 1.2/1000 live births (7). Unilateral spasticity and weakness is also common (prevalence 0.6/1000 live births) with roughly one-third of cases resulting from focal periventricular white matter lesions and one-third involving cortical or deep gray matter lesions, mainly as a result of infarcts of the middle cerebral artery. A further fifth of such cases result from brain maldevelopments, mainly focal cortical dysplasia or unilateral schizencephaly (8).

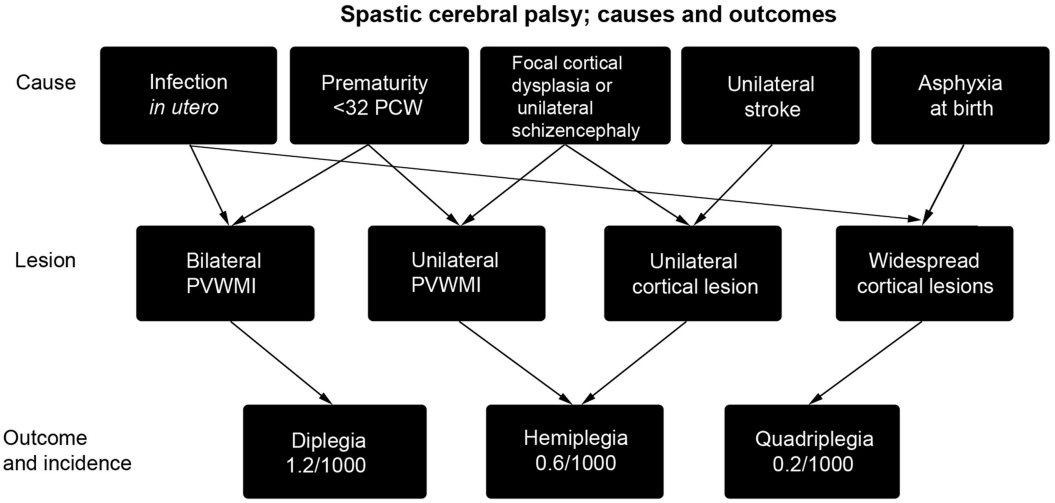

FIGURE 1 | A summary of the causes of spastic cerebral palsy, and the particular outcomes they lead to [reproduced with permission from Ref. (11)]. Asphyxia at birth may arise from prolapsed cord, intrapartum hemorrhage, uterine rupture, or maternal cardiac arrest. As arrows indicate, multiple causes may combine to produce cerebral palsy (4) and may also interact with subtle genetic variations in individuals that cause predisposition to stroke (6). PCW, post-conceptional weeks; PVWMI, periventricular white matter injury.

More severe hypoxia or anoxia at the time of birth is associated with widespread injury of white and gray matter resulting in spastic quadraparesis along with severe cognitive deficits. In all cases, there is a progressive evolution of the movement disorder over months and years. Perinatal lesions of the corticospinal system give rise to subtle but observable changes in spontaneous general movements without giving rise to the traditional neurological signs observed in older children and adults (9, 10).

PERIVENTRICULAR WHITE MATTER INJURY

Periventricular white matter injury is commonly seen in premature and low birth weight babies. It leads to lesions which range from regions of hypomyelination up to cystic lesions of the sub-cortical white matter adjacent to the external angles of the lateral ventricles (12) that largely leave the cortical gray matter intact, although cortical projection neurons may subsequently make aberrant intracortical axonal projections (13) and neuroimaging and neuropathological studies do show some reduction of cerebral cortical gray matter volume and reduced gyrification (14–16). PVWMI is the most important cause of cerebral palsy in prematurity and its incidence, along with the severity of cerebral palsy, have actually increased over time as medical advances have led to a greater survival rate for premature infants (17). Its etiology is multifactorial and possibly combinatorial, involving both prenatal and perinatal factors that may include genetic causes, ischemic-reperfusion failure, growth factor deficiency, and infection or inflammation ante- or postnatally (18, 19).

Thus age dependent regional susceptibility is a major characteristic of PVWMI with the highest susceptibility in the human brain between 24 and 32 weeks post-conceptional age (PCW); a stage of vascular development that leaves the periventricular regions at risk of hypoperfusion and hypoxia (20). Lesions occurring in PVWMI are located at the termination of major cerebral vessels in a border zone between anterior and middle and posterior cerebral arteries (21). These termination areas or "watershed areas" are located most distal from direct blood supply and are poorly vascularized (22). The temporal window during which PVWMI occurs closes between 30–32 weeks PCW, coincident with a marked increase in vascular supply to the white matter (23).

At these vulnerable stages of development, the white matter grows rapidly. This requires more energy but at the same time distance from the blood vessels is increased. The combination of these factors explains why the white matter is particularly vulnerable to asphyxia, hypoxia, ischemia, and trauma (13). The sub-cortical white matter is populated predominantly by premyelinating oligodendrocytes (24, 25) including precursor cells and immature oligodendrocytes. Such cells are more vulnerable than mature oligodendrocytes to a variety of H/I injury-related insults including glutamate receptor-mediated excitotoxicity (26, 27) and glutamate transporter malfunction (28, 29) as well as arrested development (30, 31), which may arise out of oxidative stress on the cells (32) or inhibition of differentiation by extracellular components of any astrocytic scar (33). A comparison between the timetables for oligodendrocyte production, maturation, and myelination in human and rodent forebrain is presented in **Figure 2**.

Developing white matter is vulnerable to intra-uterine infection. This can cause severely altered fetal pulmonary function and cardiovascular control, contributing to H/I brain injury, while pro-inflammatory cytokines can interact directly with various cell populations in the brain (19, 37). In particular, the external angles of the lateral ventricles, a "crossroads" site for various axonal projections, are a location for accumulation of microglia cells, which may be involved in axonal guidance but also provide a substrate for an enhanced inflammatory reaction in PVWMI (38) producing pro-inflammatory cytokines, as well as excitotoxic glutamate and free radicals (32, 39, 40). Pro-inflammatory cytokines are also able

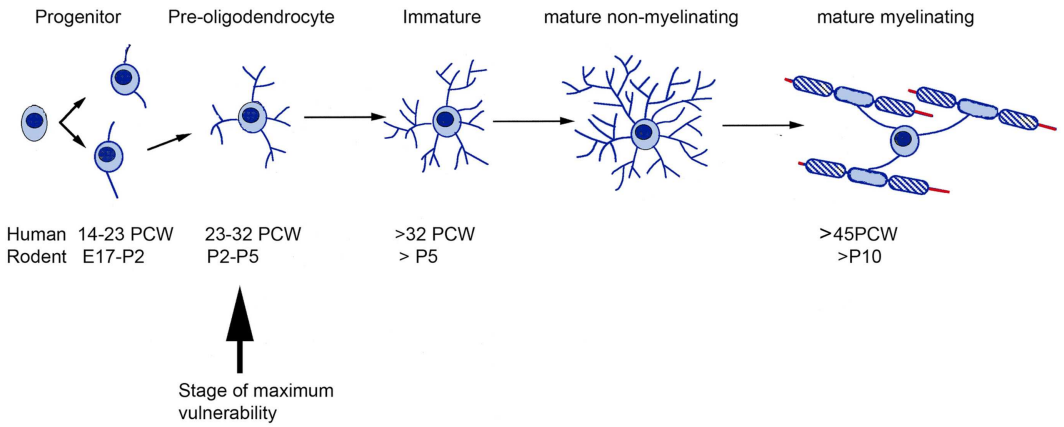

FIGURE 2 | A comparative timetable of oligodendrocyte development between rodent and human. The time of greatest vulnerability to hypoxia/ischemia (arrow) is at the pre-oligodendrocyte stage of development. Based on the information from Ref. (34–36). E, Embryonic day; PCW, post-conceptional weeks.

to disrupt glutamate homeostasis and inhibit glutamate transport in oligodendrocytes and astrocytes (29, 41).

In addition to white matter injury, the transient subplate zone of the developing human cortex peaks in size between 24 and 32 PCW (42). It is located between the periventricular white matter and the smaller, developing cortical plate and has been shown to be vulnerable to H/I injury in the preterm (43). It is relatively more mature than the cortical plate, having a better developed synaptic circuitry (44) and a higher expression of glutamate receptors making its neurons relatively more vulnerable to excitotoxic injury (45, 46). Subplate neurons play an essential role in the development of connections between thalamus and cortex and of connections within the cortex (47, 48). The time period of vulnerability to PVWMI, with its secondary damage to axon tracts and to subplate neurons, coincides with the timing of thalamocortical and corticocortical (49) and corticospinal synaptogenesis (50) and thus can be viewed as perturbing the trajectory of sensorimotor development at a crucial stage leading to aberrant development of connectivity and mapping of functions (51, 52).

PERINATAL STROKE LEADING TO SPASTIC HEMIPLEGIA

The incidence of stroke is highest in prematurely born babies compared to any other time of life and is also high for babies born at term (53). Two-thirds of children who suffer from perinatal stroke develop cerebral palsy and nine tenths of these will develop hemiplegic cerebral palsy (54). The outcome after adult onset stroke is largely determined by the extent of the initial brain injury and motor recovery occurs if a critical amount of corticospinal system function has been spared (55). However, this is not the case for a perinatal stroke and infants with a significant corticospinal projection from the infarcted cortex soon after the stroke, detected by transcranial magnetic stimulation (TMS), can still have a poor motor outcome (56). A longitudinal study has shown that in the first 24 months after stroke, progressive loss of corticospinal projections from the affected cortex may occur. Findings at 24 months were predictive of outcome; those in whom TMS failed to evoke responses in the affected limb had a poor outcome, failing to develop functional use of their paretic hand, whilst those in whom a response has been preserved had a better outcome, developing functionally useful dexterity in childhood (56).

After a unilateral stroke, although a corticospinal projection may be present, activity in the infarcted cortex is suppressed. Thus it has been proposed that surviving, but not very active, corticospinal projections may lose out in competition for spinal cord synaptic space, leading to these projections being withdrawn as their potential targets are taken over by more active ipsilateral corticospinal projections from the unaffected hemisphere and also by proprioceptive muscle afferents (51, 57).

COMPARISONS BETWEEN SPECIES

PERIVENTRICULAR ZONES AND SUBPLATE

As discussed above, hypoxic-ischemic lesions in very premature babies target the proliferative zones around the lateral ventricles, the developing white matter tracts and subplate. At what stage of development are these structures comparable to human in our animal models? In rodent, ages ranging from embryonic day (E) 18 to post-natal day (P) 7 as the time of insult have been proposed to model human lesions in the early third trimester.

White matter vulnerability is developmental regulated, and it has been related to the presence of pre-oligodendrocytes in developing axon tracts of the forebrain during the time of peak incidence of PVWMI (see Section "Periventricular White Matter Injury", **Figure 2**). In the neonatal rat, pre-oligodendrocytes are predominant in the corpus callosum and cortex between P2 and P5, whereas, immature oligodendrocytes predominate by P7 (58). Both *in vitro* and *in vivo* experiments have provided the evidence that the pre-oligodendrocytes are much more susceptible than immature oligodendrocytes to oxidative stress (59), oxygen–glucose deprivation (27), and glutamate receptor-mediated excitotoxicity (26, 60, 61). Transient synapses between growing axons and pre-oligodendrocytes play an important role in white matter development (62–64) and these are rapidly lost during hypoxic-ischemic episodes, prior to any cellular loss (65). Diffuse hypomyelination was seen in response to injections of excitotoxic ibotenic acid (IBA) into the periventricular white matter at P5 but not at P7 (66).

Therefore, most experimenters model PVWMI in rodents by delivering an H/I or excitotoxic lesion (67, 68) during the period P2–P5. At this very early stage, the corticospinal tract (CST) has reached the spinal cord but has barely begun innervating the gray matter (69). Thalamic afferents are making global, rather than lamina specific, connections throughout the cortical plate and subplate (70). Spontaneous movements, generated by bursts of activity in the spinal cord, feedback sensory information to the somatosensory cortex producing gamma oscillations followed by spindle shaped bursts of oscillatory activity (71, 72). Similar processes are occurring in human development between 24 and 32 post-conception although cortical oscillatory bursts may continue until birth (73, 74). This synchronized oscillatory network activity is proposed to drive the generation of cortical circuits (75). Thus it would appear that the period white matter vulnerability in rodents and humans is broadly comparable in terms of the stage of development of corticospinal and thalamocortical connectivity, arguably making rodents an appropriate model at this age.

The other major target for periventricular injury is the subplate, which is strikingly different in humans and rodents. In any species, the subplate is a highly dynamic compartment containing both stationary and migrating glutamatergic and GABAergic neurons, various corticopetal and corticofugal projections, glial cells, and blood vessels (48, 76, 77). In rodents, most of the subplate cells are in a thin band separating the white matter from layer 6, but some scattered cells in the upper intermediate zone are also considered to be part of the rodent subplate (78). In primates, the proportion of the subplate in relation to the rest of the cortical compartments is much greater (79). In human, the subplate zone proper becomes visible as a cell-poor/fiber-rich layer situated between the intermediate zone and cortical plate (79, 80) at around 14/15 PCW. It forms from the merging of the deepest layer of the cortical plate, with an already formed pre-subplate that contains few neurons but a differentiated neuropil featuring dendritic arborizations (81) and synapses (79), which include GABAergic elements (82) and monoaminergic innervation from the brainstem (83). This coincides with the invasion of the subplate region by thalamocortical afferents and basal forebrain afferents (84–86) as causing rapid expansion of the subplate so that it comprises a third of the cerebral wall by 16 PCW.

Birth-dating studies in rodent reveal that the subplate is among the earliest generated and earliest maturing cortical neuron population (87, 88) and in rat, becomes distinct structure from around embryonic day E16–18 (89). In contrast, in primates, neurons are continuously added to the subplate until relatively late stages of corticogenesis, including glutamatergic neurons (80, 90, 91). The subplate reaches its maximum thickness at the late second and early third trimester, and thereafter the subplate gradually decreases in size and becomes unrecognizable around the sixth post-natal month (79). The beginning of subplate neurogenesis and the arrival of the first GABAergic neurons in the subplate occur at similar stages in rodent and human (92). However, the continued addition of neurons to the primate subplate and the relatively larger proportion of the cortical wall it occupies represent major differences at later stages. Furthermore, the human subplate is compartmentalized, with neurons of different phenotypes (82, 92) and different axonal pathways (15, 77, 93) appearing in deep and superficial layers.

In summary, any lesion to the developing cortex is likely to occur at a time point when the subplate is very different in rodent and human. The human subplate will contain more glutamatergic neurons, perhaps giving greater scope for excitotoxic damage. The role of the subplate as a waiting zone for the massively increased number of intracortical fibers seen in primates will not be explored in rodent models. For instance, a recent study that explored the effect of *in utero* hypoxia at E18 on the subplate and subsequent cortical development in rodent (94) targeted the early subplate when human and rodent are more similar, but would be a model of a lesion caused during extreme prematurity in human and thus of limited clinical relevance, although otherwise of great interest from a developmental neuroscientist's perspective.

CORTICOSPINAL SYSTEM

A major factor in the development of spastic cerebral palsy is injury to the sensorimotor cortex and its sub-cortical white matter. Our ability to model cerebral palsy is crucially dependent on understanding similarities and differences in the corticospinal system function and development in human and other species. Corticospinal projections act in parallel with a number of other descending pathways and their fields of termination overlap. In addition, the sensorimotor cortex, as well as making direct connections to the spinal cord, also connects with the origins of the other descending pathways (95). The CST provides excitation/inhibition of motoneurons, along with descending control of selection, gating, and gain control of exteroreceptive and proprioceptive sensory afferent inputs, as well as mediating plasticity in spinal cord circuits (95, 96). All descending pathways function as part of a large network rather than as separate controllers of spinal cord centers, and the spinal cord, along with segmental inputs, are part of the network.

Developmental damage to the cortical component not only removes this element of motor control, but, as has been already been alluded to (see Section "Perinatal Stroke Leading to Spastic Hemiplegia"), removes an important influence on the way in which this distributed network is developing. Although it has often been proposed that the developing motor system has increased plasticity with which to compensate for these deficits (97, 98) there is also abundant evidence that aberrant plasticity leads to the increased and different symptoms seen in cerebral palsy compared to adult stroke (51, 99). Therefore, in choosing an animal model and interpreting the results of lesions we need to know how, and the extent to which, the sensorimotor cortex plays a role in the motor control network, and how it develops. A comparative timetable of development between rodent and human is shown in **Figure 3**. As the figure shows, to begin with, spinal cord and sensorimotor cortex develops independently, but at the same time as corticospinal axons begin innervate the spinal cord gray matter, ascending thalamic afferents begin to innervate layer IV of the somatosensory cortex. At this stage, damage to one element of the system, CST or subplate, perturbs development of the whole system.

Rodents have a CST that projects the full length of the spinal cord (102, 103) and is involved in fine movement control (104)

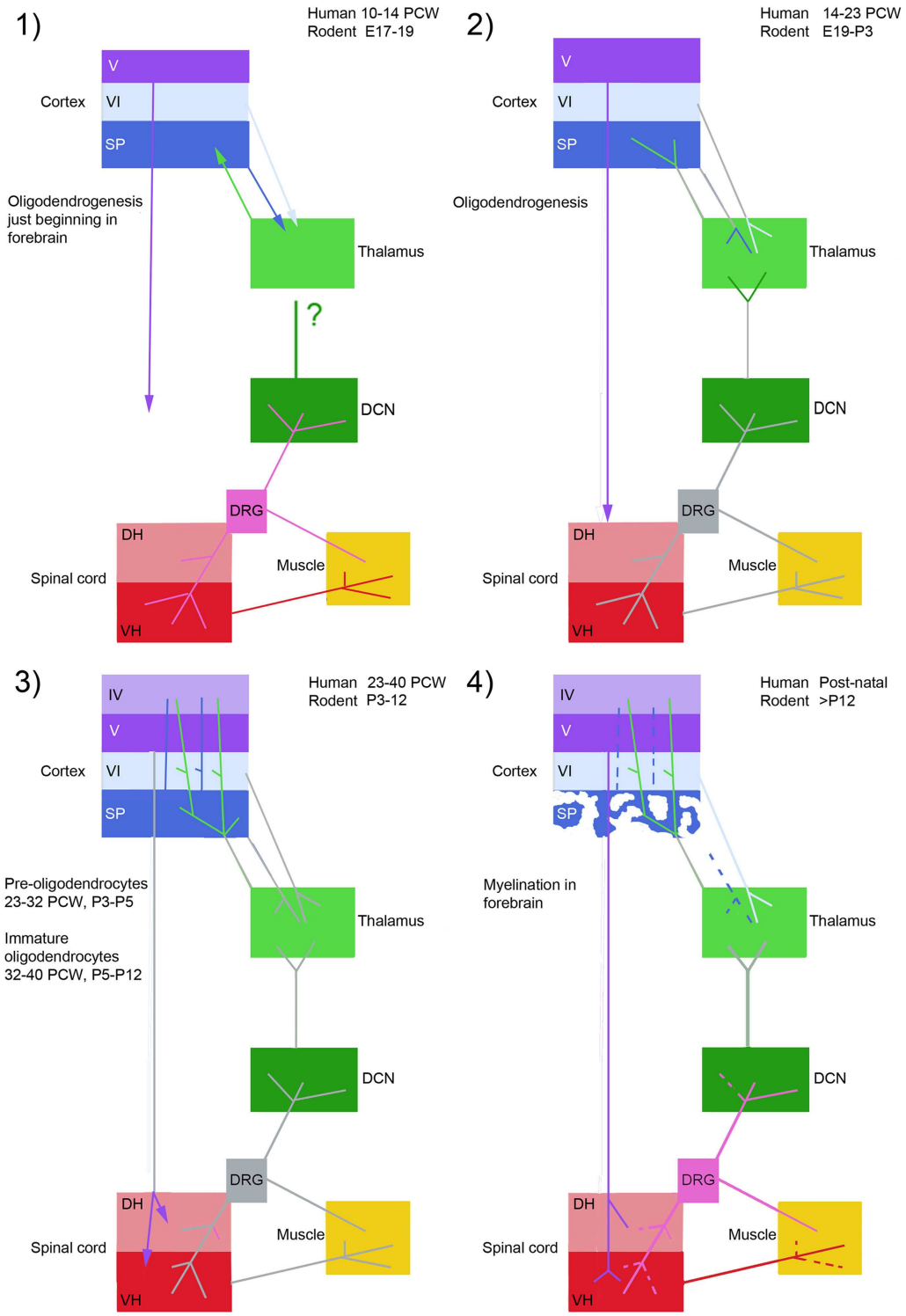

FIGURE 3 | This figure compares four stages of development of the corticospinal system in rodent and human. At stage 1 segmental circuits are connected, and local circuitry is also forming in the forebrain, but there is no connectivity between the two. Stage 2; thalamic afferents invade the subplate, and the corticospinal tract waits in the white matter to innervate the spinal cord gray matter. Stage 3; thalamic afferents innervate layer IV of the cortex at the same time as corticospinal fibers innervate the spinal cord, thus the spinal cord and sensorimotor cortex become reciprocally connected. Spindle bursts in response to spontaneous movement are recorded in somatosensory cortex. Stage 4; the subplate dissolves and corticospinal connections and muscle afferent projections are refined in the spinal cord and dorsal column nuclei. DCN, dorsal column nuclei; DH, dorsal horn; DRG, dorsal root ganglion; SP, subplate; VH, ventral horn; IV, V, VI, cortical layers. Arrows represent ingrowth of axons, dashed lines withdrawal of axon terminals. Axon projections colored gray have not changed at that stage in the figure. Based on information from Ref. (42, 50, 57, 75, 92, 100, 101).

however, the primate CST arises from a proportionally larger area of the cerebral cortex (105), possesses a fast-conducting component and the corticospinal axons are largely situated in the lateral, not dorsal, columns of the spinal cord, as they are in rodents. Kittens have also been studied as they also have the advantage of being born early in the development of the motor system, and that there is a wealth of previous research on the feline locomotor system (96).

Differences between rodents and primates in the pattern of CST terminations are both qualitative and quantitative. In rodents, the CST almost entirely projects to dorsal horn neurons and premotor spinal circuits (102, 103). In many non-human primates, such as the rhesus monkey, the projection pattern of the CST is much more complex: a significant proportion of CST fibers projects to the ventral horn, and some axons synapse directly on motoneurons, in particular those innervating hand muscles (106). In humans, this trend is even more marked (107). For example, there is a strong correlation between the number of direct connections between cortex and motor neurons and the level of manual dexterity of non-human primate species (106, 108). Rodents have very few, if any, direct connections (103, 109, 110) and this observation has been employed to explain a perceived relative lack of ability to control hand/paw musculature (111) although it has been claimed that rodents have more dexterity than is generally appreciated, which is impaired by CST or sensorimotor cortex lesions (112, 113). Similarly, damage to the CST in rhesus monkeys causes permanent deficits during stepping (114) as in humans where CST damage is severe enough to compromise independent walking (115). It has been claimed CST lesions have little effect on stepping in rodents (116) however, a more recent study have demonstrated that CST function is necessary for the avoidance of obstacles during stepping (117). In conclusion, although subtle, rodents do suffer deficits in skilled motor performance following injury to the CST, but these require subtle outcome measures to be detected.

There has also been an evolution in the role that parallel descending pathways play. In both cats and rodents, there is a prominent contralateral rubrospinal projection mostly from large neurons in the red nucleus to premotor neurons and motoneurons in the spinal cord (118–121). This is greatly reduced in macaques, which have, instead, an expanded rubro-olivary projection from small cells in the nucleus with projections from the sensorimotor cortex predominantly target the small cells (122). In human, the rubrospinal tract is greatly reduced, although still present (123, 124). Similarly, cats possess C3–C4 propriospinal interneurons that are the relays for a significant di-synaptic pathway between the cortex and motoneurons of the lower cervical cord (125) but here is no evidence for such a pathway in macaques unless inhibition in the spinal cord is greatly reduced (126). Indirect measurements have provided evidence for this pathway in humans and it may be up-regulated in patients with hemiplegia after stroke (127). However, this pathway appears not to exist in rodents (110). Cortico-reticular pathways to the spinal cord, including direct projections to motoneurons in monkeys (128, 129) have been described, although it is worth noting that even in rodents there are inter species differences with mice having a much weaker excitatory pathway than rats (110). Exactly what plasticity may occur in unlesioned descending pathways is species dependent, and this needs to be taken into account when interpreting animal models.

Another important consideration is the extent of the ipsilateral CST. In macaques it is quite large; 13% of all corticospinal axons fail to decussate in the medulla (130) and this is similar to the human CST (131) whereas in rodents only 2–4% remain uncrossed (132). The adult ipsilateral projection is also similarly small in the cat (133). To confuse matters more, in monkeys there are bilateral projections and fibers crossing from the ipsilateral to contralateral side at the spinal cord segmental level, as well as contralateral axons re-crossing to terminate on the ipsilateral side (130, 134) but in rodents nearly all corticospinal axon terminate without crossing the spinal cord midline (135, 136). However, it should be born in mind that ipsilateral corticospinal connections in the monkey cervical spinal cord are different from contralateral projections as they fail to make monosynaptic connections with motoneurons (137).

Plasticity in the ipsilateral tract following a perinatal hemiplegic stroke could provide a gateway to improving function in the affected limbs. Surprisingly, there is evidence for an extensive transient ipsilateral projection in humans, where, in the newborn, TMS is as likely to produce ipsilateral contractions in arm muscles as it is contralateral muscles, only with a shorter latency, suggesting a direct projection (56). These ipsilateral projections are down-regulated during normal post-natal development, however in patients with hemiplegia derived from a pre- or perinatal lesion, or developmental malformation, these ipsilateral connections are retained (56, 138, 139), although they confer no functional advantage (56). Hypertrophy of the pyramid contralateral to the lesion has been interpreted as showing that the fibers are retained projections from neurons in the intact hemisphere normally lost during development (56). To what extent this can be modeled in animals is discussed in Section "Modelling Corticospinal Plasticity."

Fast onset, low threshold, and aberrant reflex pathways are observed in spastic cerebral palsy sufferers (140, 141) may result from retention of developmental reflex pathways in the absence of corticospinal input at a crucial stage of development (57). In human and rodents alike the excitation threshold of stretch reflexes increases with age (142, 143). This may partly be because muscle afferents first target the cell bodies and proximal dendrites of motoneurons in both rodent and human (144–147) although, in maturity, these afferents principally target more distal dendritic sites (148).

Activation of the stretch reflex in the biceps brachii of a newborn human also results in fast heteronymous excitation of antagonist muscles such as triceps brachii, providing evidence for the existence of superfluous connectivity that is presumably eliminated later in development (143). However, in rodents much research suggests that muscle afferents innervate homonymous and synergistic motoneurons with a high degree of accuracy from the outset (149–151). Nevertheless, patterns of muscle afferent innervation change with development in the rodent ventral horn (146) and cuneate nucleus (101) and in the intermediate gray (152) of the kitten spinal cord. Therefore, it may be possible to study some aspects of aberrant spinal cord development in response to cortical lesion, but the high degree of spasticity and aberrant reflex formation observed in humans is not be substantially reproduced in rodents.

CRITICAL EXAMINATION OF ANIMAL MODELS IN USE

MODELS OF PERIVENTRICULAR WHITE MATTER INJURY

Based on the various risk factors discussed in Section "Cerebral Palsy in Humans," various animal models have been developed in different species but mostly rodents, including models of hypoperfusion and models using infectious agents, bacterial products, or excitotoxic insults. These varied approaches were extensively reviewed by Hagberg et al. (67) and their recommendations have strongly influenced the field ever since. Approaches used in rodents fall into two main classes; firstly, the induction of H/I by the maintenance in a hypoxic environment for a period of time, coupled with unilateral ligation or cauterization of the common carotid artery, the Rice–Vannucci model, which has been use for over 30 years and has the advantage of being extremely well characterized (153). The drawback is that although the lesion is reproducible and bears some resemblance to lesions observed in affected infants, the method for inducing it is artificial. Also, this approach is generally employed at P7 or slightly later, and as discussed in Section "Cerebral Palsy in Humans," the period of peak oligodendrocyte vulnerability occurs a little earlier (**Figure 2**). Thus, although the Rice–Vannucci method recently has been applied at earlier ages [e.g., Ref. (31, 154)] because of the difficulty of employing the Rice–Vannucci approach at younger ages other approaches involving modeling the consequences of hypoxia have also been employed including intracerebral injection of excitotoxic agents (66, 68, 155, 156) or agents causing oxidative stress (157). Hypoxia on its own has also been employed, for instance gestational hypoxia between E5 and E20 in rats induced white matter damage due to a local inflammatory response and oxidative stress linked to re-oxygenation during the perinatal period (158) however, the relevance of this model to most cases of cerebral palsy is not clear.

Systemic or intracerebral injection of inflammatory agents between P3 and P7 has also been employed (159–161). These approaches again yield reproducible lesions but only model some aspects of the human condition. Because intra-uterine inflammation may be a significant contributing factor to brain injury leading to cerebral palsy, many animal models have been developed in which intra-uterine inflammation is instigated in rodents and rabbits prior to birth [reviewed by Burd et al. (162)]. The significant drawback with these experiments is that they are instigated very early in development, as the species are born at a very premature stage of development compared to humans (see **Figure 2**). For instance, some experiments have taken place at E9–10 in mouse (163–165) at a time when neocorticogenesis is only just beginning [7–8 PCW in human (166)] and this really only suitable for modeling proposed neurogenesis and cell migration deficits seen in neurodevelopmental disorders such as autism or schizophrenia. Even studies toward the end of rodent gestation (167–169) or rabbit (170, 171) are modeling extreme prematurity, that is, halfway through the second trimester (166) and therefore of limited relevance to most cases of cerebral palsy.

The purpose of developing these models has included both testing early interventions for preventing or reducing PVWMI, and discovering other factors that exacerbate the condition. For instance, a model of PVWMI induced by intracerebral excitotoxin injection at P5 has been shown to be exacerbated by additional systemically administered pro-inflammatory cytokines and interleukin-9 (172) helping to establish the multifactorial nature of the condition. Similarly, excitotoxic lesions were significantly worsened in mouse pups exposed to gestational stress caused by a significant rise of circulating corticosterone levels both in pregnant mothers and in newborn pups, acting through glucocorticoid receptors (173). Using transgenic technology, the widely expressed kinase GRK2 has been implicated in protecting white matter against H/I injury (174) suggesting that genetic variability between individuals may contribute to the severity of perinatal brain damage. A recent study has revealed a novel, gender-specific protective role for innate immune receptor signaling in a mouse model of neonatal hypoxic-ischemic brain injury (175) revealing another potential source of variability in injury severity.

Testing protective interventions has been carried out in many and varied studies. For instance, the extent of injury has been reduced by administration of glutamate antagonists (26) including successful magnesium sulfate as a blocker of NMDA receptor channels (155, 176) leading to clinical trials of this approach, although not, as yet, with any convincing evidence of beneficial effects (177). A variety of other agents have been trialed pre-clinically with some promise of efficacy, including vasoactive intestinal polypeptide and melatonin, which act by modulating second messenger systems (178, 179). Stem cell therapies have been tested pre-clinically, which may modulate the inflammatory response and/or stimulate host production of new oligodendrocytes (180–185).

The antibiotic minocycline, which also inhibits the activity of microglia, has been extensively tested and reduces white matter damage and brain lesion size [e.g., Ref. (186–188)]. However, minocycline studies also provide a lesson in the problems of scaling up pre-clinical trials in rodents to human as explained by Buller et al. (189). Preconditioning dosing strategies may be more beneficial, however administration post-insult has more clinical relevance, as a diagnosis of perinatal HI in the neonate is often not made until 3 days after birth. Routes of administration appropriate for babies have not undergone trials. Large single doses exacerbate injury in mice (190) but this may be strain specific. Repeated doses of the drug appear to be more effective (188) but it is difficult to predict the length of treatment required when glial cells mature in a matter of days in rodents but over months in humans, bearing in mind the detrimental effects of tetracycline antibiotics on growth of bones and teeth (191).

As well as differences in maturation time compared to the gyrencephalic human brain, rodents also have a substantially smaller proportion of sub-cortical white matter, substantial differences in cerebral blood flow and metabolism and a greater susceptibility to gray matter injury in response to white matter lesions (153, 192). Thus, the fetal sheep has been proposed as an alternative model for a number of reasons. It is possible to perform experiments and make repeated measurements *in utero*. The stage of development of the ovine fetus at 95 days post-conception shows strong similarity with the early third trimester human, both in terms of oligodendrocyte development (193) and general brain development including in terms of the completion of neurogenesis, the onset of cerebral sulcation, and the detection of the cortical component of somatosensory evoked potentials (192). Melatonin therapy has been pre-clinically tested, with success, in sheep (194). Adaptive brain shut down and neuroinflammation have also been

studied in the near term ovine fetus (195, 196). However, a significant drawback is that although sheep might provide a good model of white matter damage, they provide a poor model of corticospinal function as the CST fails to project below the upper cervical level (197) and no protocols have been developed for neurobehavioral studies of sheep receiving preterm lesions.

MODELS OF PERINATAL ISCHEMIC STROKE

In human neonates, perinatal arterial ischemic stroke (PIS) events occur mostly in the middle cerebral artery (198, 199). Therefore, focal MCAO models reflect the vascular distribution seen in human neonates with ischemic stroke rather than other H/I models that more accurately model PVWMI (see above). The heterogeneous nature of PIS in human leads to two types of studies. Some investigators have used permanent focal MCAO for animal models, while others apply transient occlusion that allows reperfusion for occluded vessels. The pathology of both types is similar although the injury pattern and severity of brain injury differ. A permanent occlusion results in a severe ischemic injury accompanied by necrosis, whereas transient occlusion can produce a lower injury severity, depending on the occlusion duration, accompanied by apoptosis (200, 201). There is also apoptotic like cell death during the first 24 h in permanent occlusion models (202). After introducing these types of lesion to rat pups, two zones of ischemic injury occur; a central, necrotic injury zone with little scope for recovery, and a penumbra where apoptotic cell death is more usually seen and there is some scope for rescuing the tissue (200, 201, 203, 204).

Studies that used transient MCAO (200, 205–207) claim that their model reflects neonatal PIS since reperfusion mimics what happens to neonates when circulation is permitted by collateral circulation to the penumbral part of the ischemic lesion (208). On the other hand, studies not involving reperfusion in their MCAO model argue that there is no consistency in reperfusion among patients (209). The Left middle cerebral artery is most commonly occluded in neonatal ischemic stroke (198, 210) and so is most commonly targeted in animal models. The internal carotid artery is catheterized by monofilament suture to occlude the middle cerebral artery permanently by retaining the filament, or temporarily by removing it at the desired time (211).

This approach was first applied to young rats (P 14–18) by Ashwal et al. (205) to cause transient occlusion at the proximal middle cerebral artery followed by reperfusion. Cytotoxic edema occurred in the ischemic region immediately after the occlusion, then severe injury in a similar region occurred after reperfusion (200). A study that used high-field MRI over a 28-day period post-lesion demonstrated that transient filament MCAO models induce infarction with maximum volume at day 1–3 post-occlusion (207). Three hours of occlusion resulted in infarcts that included the striatum and affected 40–50% of the whole hemisphere and may resemble human stroke (205). However, this method produced unacceptably high mortality rates where only 21% of pups survived for more than 28 days (207). Animal welfare concerns apart, this does not allow for long term assessment of treatment outcomes. Interestingly, transient occlusion of the common carotid artery for 60–90 min, combined with permanent ligation of the middle cerebral artery produced only neocortical injury (203) however, whether occlusion of arteries external to the cranium really models human strokes is questionable. Nevertheless, such models have a lower mortality rate and can cause sensorimotor and cognitive impairments in early adulthood such as postural asymmetry, motor incoordination, and cognitive impairments, although the lesion site is small by this age (204).

The introduction of an embolus into the MCA, guided from the CCA or ECA with a filament, was pioneered by Derugin et al. (206) and further refined by individualizing embolus size to the rat's size (202). It was claimed that the infarction pattern in their model mimics that of the MRI pattern for the human neonate (212). Infarcts in this model are located in the cortex and the striatum, and the infarcted area in the cortex is 51–56% of the ipsilateral hemisphere in the forebrain and no mortality during this time period (202).

Another approach is to ligate or electro-coagulate the distal middle cerebral artery, approached following a craniotomy, to produce permanent occlusion. MCA ligation performed at the level of inferior cerebral vein in mature and immature rats fails to cause an infarction in all animals (203, 213, 214). If applied at the level of the olfactory tract, infarction resulted in 13% of rats; occlusion at the MCA origin caused infarction in 67% of rats. To achieve 100% of rats with cerebral injury, ligation 3–6 mm along the MCA starting from its origin or proximal to the olfactory tract to the level of inferior cerebral vein is required, which would include all supplying arteries from the proximal to distal portion of MCA (214). Recently, this model was applied in neonatal Cb-17 mice producing selective and consistent cortical injury, mild corpus callosum atrophy, and mild thalamic injury similar to what is seen in infant stroke and leading to significant sensorimotor defects (209). The method is highly reproducible in this mouse strain; the operation requires $<$15 min and a 100% survival rate is reported. Reproducibility may be due to the small variation in cerebrovascular structure observed in these mice (215) and it advised that rodent strains with a robust collateral blood supply to sensorimotor areas, for instance Wistar rats, are avoided when contemplating these experiments (208, 216). Strain can also strongly influence the ischemic injury pattern, for instance, CD1 mice after carotid ligation on P12, are more vulnerable to epilepsy than C57Bl/6 mice, as are the C3Heb/FeJ strain (217, 218).

Alternatively, thrombosis can be induced by injecting the vascular system with a photosensitive dye and exposing blood vessels to light resulting in permanent focal ischemia (219). Permanent occlusion was produced in piglets by exposing the MCA. Severe reduction in cerebral blood flow and gray and white matter injury with 7.1–12.3% infarction volume of ipsilateral hemisphere occurred in this model (220). This has also been applied in 7-day old-rats causing direct injury to the sensorimotor cortex (221). As laser exposure duration increased, so did severity and size of the injury and the deficit in motor performance (221). Thus, infarction volume can be controlled according to the exposure time. In addition it is a non-invasive method with low mortality rate (220, 221). However, the pathogenesis of this focal ischemic infarction is of debatable relevance to human neonatal stroke.

The events of perinatal ischemia are suggested to occur any time over a period of 20 weeks that spans late fetal and early neonatal life (222). Thus human perinatal stroke are classified according

to the infant age when diagnosis is made as well as radiological assessment patterns of injury (199, 222). However, the first week of life is the main period when PIS will occur (199). The use of animal models, mainly rodent, to reflect ischemic stroke in the perinatal human period depends on matching the appropriate age between human neonate and animal models by correlating neuronal events that occur during maturation. Correlating human full term to model post-natal age (P) is an area of conflict in the literature. Based on different criteria, authors claimed that human term corresponds to either P7 (223) or P8–14 of rodent age [white matter development (67); Corticospinal system development (57); and EEG maturation (224)]. Several of the earlier studies discussed above have used P7 rodents (200, 202–204, 206, 221) based on Hagberg et al. (223). Other studies have used a more appropriate age either because of the difficulty of performing experiments in younger animals (205, 207) or following Hagberg et al. (67) for example Tsuji et al. (209).

Finally, it should be born in mind that an infarction that destroys the sensorimotor cortex may not be required to model cerebral palsy. Eyre et al. (56) demonstrated that in human developmental hemiplegia during the earliest stages, a corticospinal projection is still present, which fails to develop and is withdrawn. Therefore, the aim may be to induce a degree of hypoxia that delays maturation of the cortical tissue rather than destroys it completely, and it may be that more detailed measures of outcomes are required in our animal models than the presence or absence of tissue.

MEASURING OUTCOMES WITH MODELS OF PVWMI AND PIS

A problem with interpreting all animal models of PVWM and PIS is the diversity of outcomes measured. We have surveyed a sample of studies in rodents, taking as our sample the 36 studies cited in Sections "Models of Periventricular White Matter Injury" and "Models of Perinatal Ischemic Stroke" above. The results are summarized in **Figure 4**. The majority of studies (56%) measured the lesion size within a week of the insult, but only 36% measured the lesion size in the longer term, either by MRI or histology. Less than a quarter of studies investigated changes in molecular markers, such as markers of apoptosis, gliosis, or myelination, in either the short or long term. Behavioral testing was even rarer. Testing of sensorimotor function was most common, being carried out in a quarter of studies, but cognition or anxiety has also been measured

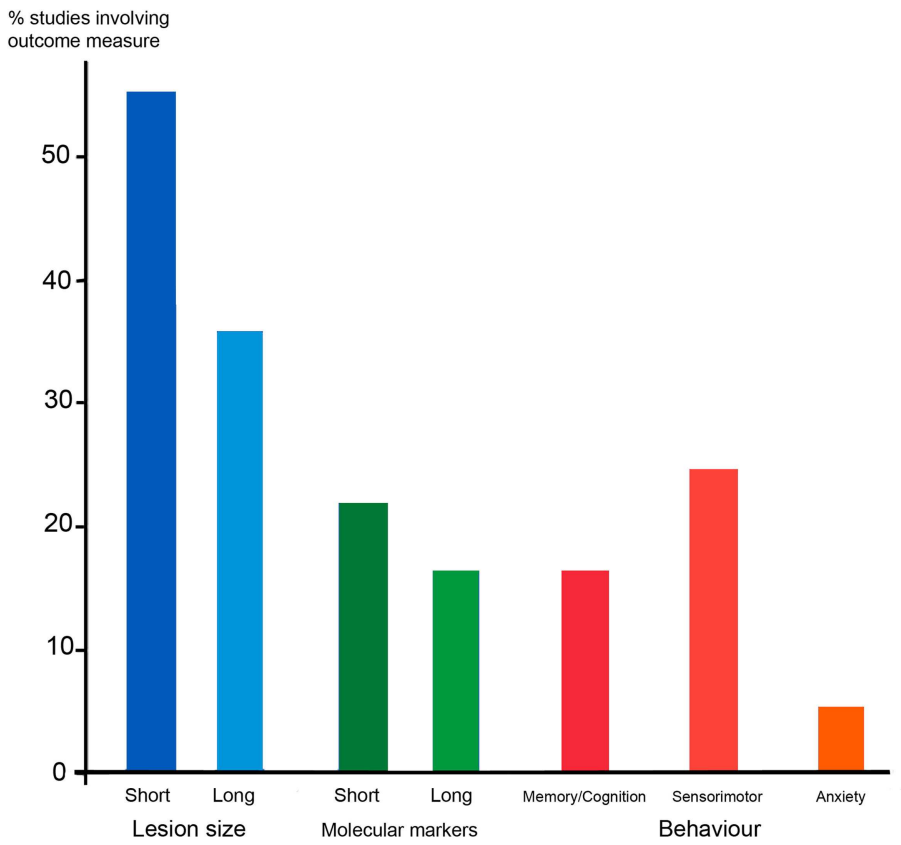

FIGURE 4 | The outcome measures employed in a sample of 36 rodent studies that modeled PVWMI or PIS, some of which involved experimental therapies. Blue columns depict the proportion of studies that studied lesion size in the short term (within a week) or in the longer term, either using MRI, or histology. Green shows studies of changes in molecular markers in response to lesions, e.g., markers of apoptosis, myelin, and gliosis. Red/orange shows behavioral testing in adolescent or adult animals following perinatal lesions. These are divided into tests of memory and cognition (e.g., mazes) sensorimotor (e.g., rotarod, reaching, and ladder walking) and anxiety (open field). The 36 studies sampled are those involving rodents cited in Sections "Models of Periventricular White Matter Injury" and "Models of Perinatal Ischemic Stroke."

in around 17 or 6% of studies, respectively. Tests for sensorimotor function employed are not necessarily very specific tests for corticospinal function, often consisting of observing the righting reflex or rotarod performance, and rarely testing limb placement or reaching skill.

Thus it appears for most researchers, the aim is to show a reduction in brain damage inflicted by whatever lesion is employed, sometimes simply by measuring the size of the damaged area, or sometimes the extent of cell death or demyelination, often just in the short term. Of course, any treatments that can be proven to ameliorate the effects of H/I, if given early, are of value. Also, cerebral palsy is not the only, or even the most common, outcome of early brain injury and it is important to access the effects on brain function other than sensorimotor co-ordination. But as has been discussed above, and will be further explored below, the mal-development of the sensorimotor system following a lesion is protracted and complicated, and animal experiments designed to model cerebral palsy must try and find ways of addressing this problem. It is paradoxical that, in human, we have long been adept at recognizing and quantifying the neurological symptoms of cerebral palsy, and only more recently have been attempting to measure the more subtle signs of deficits in cognition and attention. In animal models, it has so far been easier to measure lesion size, or standard behavioral tests such rotarod, water maze, and open field. Evidence of corticospinal deficits is harder to observe and test for, and this is the topic of the next section.

MODELING CORTICOSPINAL PLASTICITY

Spastic cerebral palsy is primarily a lesion of the CST, which results in secondary maldevelopments of related circuitry, which may include a retained ipsilateral tract and aberrant development of spinal reflex pathways (see Section "Perinatal Stroke Leading to Spastic Hemiplegia"). Might it be possible to gain useful understanding of these processes by making a controlled lesion of the sensorimotor cortex that do not necessarily mimic the injuries observed in a clinical setting? Such approaches have been adopted including aspiration of brain tissue, prolonged inhibition of areas of cortex by slow release of pharmacological agents, or genetic ablation of corticofugal tracts.

An increased ipsilateral projection has been reported following developmental unilateral lesions in animal models but the nature of the projection varies depending on the timing of the lesion and the species involved. For instance, in rodents it appears that lesions made in the first week of birth, when the majority of the corticospinal fibers are growing into the spinal cord (**Figure 3**) results in an enlarged ipsilateral projections that predominantly comprise a non-decussating pathway, or a double decussating pathway (132, 225–227). However lesions at P7 or later tend to cause branching of fibers to innervate both sides of the spinal cord (228, 229). There is no evidence for a transient ipsilateral CST in development that is proportionally larger than in maturity, in either developing rodents (230) or monkeys (134) although as the projection from cortex to spinal cord is generally from a larger proportion of the cortical surface in development than in maturity, there may still be a proportionate withdrawal of ipsilateral axons. On the other hand, in kittens corticospinal fibers initially branch and bilaterally innervate the spinal cord (231). Under normal circumstances, the transient ipsilateral projection is withdrawn whereas the contralateral projection expands and reinforces its synaptic connections (133, 231). However, the ipsilateral projection can be maintained by removing the competing contralateral projection (232) or blocking its activity pharmacologically by continuously infusing the gamma-aminobutyric acid (GABA)-agonist drug muscimol within the developing motor cortex (233). Neural inactivation is performed between post-natal weeks 5 and 7, a developmental period during which most transient dorsoventral and ipsilateral terminations are eliminated (233, 234).

Martin and colleagues have used their unilateral cortical inactivation model in kittens to test two therapeutic strategies. Firstly, the affected CST was electrically stimulated daily over three weeks between post-natal weeks 8 and 11 (235) secondly the previously uninvolved contralateral cortex was chronically inhibited at this time (236). Both methods restored and strengthened contralateral CST connections to their normal spinal targets in the intermediate gray matter and reduced aberrant ipsilateral connections. They also led to motor recovery in a visually guided motor task. This suggests that it is balancing activity in the two competing tracts that leads to correct distribution of corticospinal inputs, not the amount of activity *per se*. Their studies were extended to non-invasive behavioral approaches mimicking potential interventions in infants (237) involving restraint of the non-involved limb with or without reach training in kittens or young cats (238). Interestingly, all three interventions restored normal contralateral corticospinal termination patterns but did not reduce aberrant ipsilateral connectivity. Only limb restraint combined with reach training restored behavior. This showed that factors additional to restoring CST connectivity contribute to motor recovery. These include re-establishing a motor map, which was only achieved with reach training.

Although these experiments in kittens appear to give useful pointers to therapies for early interventions in hemiplegic cerebral palsy, the situation as hypothesized in humans requires the presence of a large transient *unbranched* ipsilateral projection that is retained following a unilateral lesion (56, 239). Possibly any human transient ipsilateral projection is actually quite small but is still able to excite motoneurons directly, owing to the greater excitability of immature motoneurons (240) in which case rodents receiving a lesion before post-natal day 7 (which have unbranched ipsilateral projections, see above) may provide an accurate model. Alternatively, bilateral excitation of motoneurons from motor cortex may result from up-regulation of a fast pathway via cortico-reticular synapses, as reticular neurons bilaterally innervate motor columns including those innervating hand or paw muscles (109, 241, 242). This form of plasticity has yet to be adequately explored in developmental models (243). Interestingly, following hemi-decortication in rat at P5, aberrant connections were formed from the surviving motor cortex to contralateral red nucleus, superior colliculus, pontine nuclei, and the ipsilateral dorsal column nucleus and cervical spinal cord, which preserved forelimb function, but no aberrant projection to reticulospinal neurons was seen (244) perhaps because a bilateral corticoreticular projection is already present.

Simple lesion experiments have explored the extent to which normal development of intrinsic spinal cord circuitry, which

extends beyond the period of CST innervation (57) depends upon a functional CST. Unilateral lesions to the sensorimotor cortex (245) or spinal cord transection during development (246) in rodents leads to retention of muscle afferents in the ventral horn and strengthened segmental reflex pathways. This is possibly analogous to the fast onset, low threshold, and aberrant reflex pathways that are observed in spastic cerebral palsy sufferers (141). Both muscimol blockade, and lesioning of the sensorimotor cortex unilaterally at P7, when the CST begins to innervate spinal cord gray matter (**Figure 3**) prevented the normal up-regulation of expression of the activity dependent marker parvalbumin in spinal cord neurons contralaterally (245, 247, 248) in rat. A recent study in mouse, in which corticospinal input was removed entirely by genetic ablation of all cortifugal outputs, did not result in loss of spinal cord parvalbumin expression (249). This might be explained by species differences, but it seems possible that an imbalance in activity, rather total loss of inputs, is required to cause some alterations in gene expression. Changes were seen in other interneuron subgroups and in motoneurons, including increased detection of cholinergic interneurons (249).

An increase in spinal cholinergic interneurons between 4 and 8 weeks postnatally in kittens is another late developmental event coincident with the re-organization of corticospinal input (250). Inactivation of the developing CST, and resulting motor impairments, significantly reduces the number of spinal cholinergic interneurons unilaterally, again highlighting possible differences between unilateral inactivation and total genetic ablation. Constraint combined with early reach training resulted in increases in number of cholinergic interneurons on the injured side of the spinal cord, far more than constraint alone or in combination with late reach training. Thus, behavioral recovery was associated with the substantially larger cholinergic interneuron response (238). Because these spinal interneurons are excitatory, they may augment the effect of CST input to spinal cord circuitry. What is required now is evidence that cholinergic interneurons play a role in human spinal cord function and development.

CONCLUSION

When considering the outcome of testing experimental therapies for cerebral palsy in animal models it is important to ask a number of questions. Firstly, what type of cerebral palsy are we modeling? As this review has shown, the timing and nature of the lesion can be varied to model different types. Secondly, are we causing behavioral deficits typical of the human condition? Rodents do not suffer spasticity or severe locomotor impairment in response to sensorimotor cortex lesions, but there is evidence of subtle, CST dependent sensorimotor deficits that can be quantified. This leads onto the third point, is re-organization of the CST the same in our models following lesion compared to human? It is clear that primate CST organization is quite different from rodents or kittens, and modeling, for instance, ipsilateral pathway plasticity is fraught with difficulty. Finally, one of the trickiest problems with rodents is the rapid development of nervous system, which can take place in the time it takes for post-lesion inflammatory processes to take place, which poses the question can we intervene quickly enough in rodent models to change the course of maldevelopments in the motor system?

It might seem that a serious pre-clinical trial of a therapy ought to include non-human primate experiments, and yet there is only one model of perinatal H/I injury in primates that has been developed, to our knowledge. This involved focused lesions to the visual cortex caused by injection of endothelin to constrict blood vessels in the P14 marmoset (251), which caused similar anatomical and cellular pathology to that observed in post-ischemic humans at a stage of visual cortex development equivalent to 3–5 months postnatally in the human. However, very little is known about sensorimotor cortex/CST development in any primate species, and so knowing when to carry out lesions would be difficult. CST ingrowth into the ventral horn and development corticomotoneuronal synapses occurs postnatally in macaque (252, 253) but by concentrating on the elaboration of corticomotoneuronal connections to hand muscles originating from specific areas of the motor cortex, these studies ignored the higher density of corticospinally projecting neurons, coming from a larger area of cortex, in the neonate compared to the adult, as detected by retrograde tracing experiments (134). Thus a whole process of corticospinal axon elaboration and refinement, including elimination of transient projections including ipsilateral axons and projections from non-motor areas, as has been proposed for human development from indirect observations (51, 254) may, or may not, be present in the non-human primate.

As always, more research is needed, but the considerable difficulties of doing even basic research on motor development in non-human primates, let alone using them for neonatal lesion studies, would seem to make it unlikely that this line of research will be frequently taken in the future, in which case it is vital we understand the limitations of translating pre-clinical research in rodent and other species to human cerebral palsy. We hope this review may be of some help in making those judgments.

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Developmental dynamics of radial vulnerability in the cerebral compartments in preterm infants and neonates

*Ivica Kostović[1], Mirna Kostović-Srzentić[2], Vesna Benjak[3], Nataša Jovanov-Milošević[1] and Milan Radoš[1]**

[1] Croatian Institute for Brain Research, University of Zagreb School of Medicine, Zagreb, Croatia
[2] Department of Health Psychology, University of Applied Health Sciences, Zagreb, Croatia
[3] Department of Pediatrics, Clinical Hospital Center Zagreb, University of Zagreb School of Medicine, Zagreb, Croatia

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Gavin John Clowry, Newcastle University, UK
Kathryn McFadden, University of Pittsburgh, USA

***Correspondence:**
Milan Radoš, Croatian Institute for Brain Research, University of Zagreb School of Medicine, Šalata 12, Zagreb 10000, Croatia
e-mail: mrados3@yahoo.com

The developmental vulnerability of different classes of axonal pathways in preterm white matter is not known. We propose that laminar compartments of the developing cerebral wall serve as spatial framework for axonal growth and evaluate potential of anatomical landmarks for understanding reorganization of the cerebral wall after perinatal lesions. The 3-T MRI (*in vivo*) and histological analysis were performed in a series of cases ranging from 22 postconceptional weeks to 3 years. For the follow-up scans, three groups of children (control, normotypic, and preterms with lesions) were examined at the term equivalent age and after the first year of life. MRI and histological abnormalities were analyzed in the following compartments: (a) periventricular, with periventricular fiber system; (b) intermediate, with periventricular crossroads, sagittal strata, and centrum semiovale; (c) superficial, composed of gyral white matter, subplate, and cortical plate. Vulnerability of thalamocortical pathways within the crossroads and sagittal strata seems to be characteristic for early preterms, while vulnerability of long association pathways in the centrum semiovale seems to be predominant feature of late preterms. The structural indicator of the lesion of the long association pathways is the loss of delineation between centrum semiovale and subplate remnant, which is possible substrate of the diffuse periventricular leukomalacia. The enhanced difference in MR signal intensity of centrum semiovale and subplate remnant, observed in damaged children after first year, we interpret as structural plasticity of intact short cortico-cortical fibers, which grow postnatally through U-zones and enter the cortex through the subplate remnant. Our findings indicate that radial distribution of MRI signal abnormalities in the cerebral compartments may be related to lesion of different classes of axonal pathways and have prognostic value for predicting the likely outcome of prenatal and perinatal lesions.

Keywords: white matter injury, subplate, transient cerebral compartments, radial vulnerability, preterm infants, corridors of axonal growth

INTRODUCTION

The process of complex growth of white matter tracts (outgrowth of axons, pathway finding, decision making, axonal guidance, waiting periods, target selection, and in growth in the cortical plate) (1–8) occur within transient cellular compartments of the fetal cerebral wall (9, 10) and different segments of the fetal white matter (11, 12). Other neurogenetic events (proliferation, migration, molecular specification, differentiation of dendrites, synaptogenesis, gliogenesis, myelination, and cell death) also take place within the same transient laminar compartments (9, 10, 13, 14). Thus, laminar compartments provide a framework for various cellular interactions important for axonal growth and formation of axonal trajectories. In humans, the process of growth and target finding of long projection and associative axonal pathways occurs predominantly during the second half of gestation (3, 8, 10, 12, 15–17). During this period, there is sequential and partially overlapping growth of thalamocortical, commissural, and associative pathways within transient laminar compartments and sagittal axonal strata (18). Although there is significant overlap in growth of different classes of axonal pathways, there is a period of an increased growth rate for every class of afferents (3, 4, 19–22). For example, the period between 22 and 26 postconceptional weeks (PCW) is essential for terminal phases of growth of massive thalamocortical pathways and their relocation from the subplate into the cortical plate (3, 10, 15, 16). The period around 28 PCW is characterized by simultaneous growth of callosal and long associative pathways within the subplate (4, 8, 12, 23, 24). The period after 34 PCW is dominated by growth of long associative cortical pathways in parallel with the formation of secondary cortical gyri (4, 8, 19, 24–29).

As demonstrated previously, axons are more vulnerable to hypoxia ischemia and other pathogenetic factors such as periventricular hemorrhages during the period of intensive growth (6, 30–39). Therefore, transient compartments that predominantly contain growing axonal pathways (fetal white matter) are the most vulnerable cellular compartments in the preterm brain (36, 37, 40). The increased vulnerability during increased growth rate of axonal pathways is consistent with an extensive body of evidence

showing that white matter injury is predominant pathology during the early third trimester (30, 31, 41–45). However, a recent review (30) suggested that, during preterm and term period, both white matter and neuronal "gray" matter are vulnerable to etiological factors, such as hypoxia ischemia. Other studies provided further evidence on the involvement of cortical "gray" in the injury of the preterm brain (46–50). In this context, the most intriguing seems to be the vulnerability of the subplate, which is the site of the earliest synaptic cortical activity and the most prominent compartment of the cerebral wall in the preterm infant. The subplate contains growing axons, postmigratory neurons, synapses, and glia (23) and is prospective target for hypoxia ischemia (30, 31, 51–53).

The presence of well defined laminar architecture of cerebral wall (23, 54) as well as defined fetal white matter segments in late fetuses (11, 18) offers a unique opportunity to study spatial parameters of selective vulnerability of different, radially arranged cerebral compartments and related growing axonal pathways. Modern imaging studies using both conventional and diffusion techniques open new vistas in study of lesions of different compartments of cerebral wall (12, 25, 26, 28, 30, 55–58). It was proposed that prenatal lesions of developmentally important transient cellular compartments imply subsequent developmental reorganization of the cerebral cortex (31, 34, 51, 53, 59–62). The imaging studies have shown that pathologies seen after ischemia and hemorrhage show differences, which partially depend on the depth location within the different segments of the cerebral white matter (11, 12, 28, 31, 36, 42, 44, 53, 63, 64). However, we still lack a detailed knowledge on the vulnerability of different classes of axonal pathways within the laminar compartments and on the vulnerability of different segments of the fetal white matter along the radial axis of the cerebral wall. Thus, developmental vulnerability of modulatory, projection, commissural, long and short association pathways, and intracortical fibers, their topographical distribution, and role in developmental reorganization and structural MR correlates remain poorly understood. In addition, the correlation with disturbances of other developmental events (proliferation, migration, synaptogenesis, dendritogenesis, myelination, and cell death) remains largely unknown.

The first step in the analysis of vulnerability of transient cerebral compartments and related cell classes is their histological delineation and elucidation of their developmental history. Based on our preview studies on laminar organization and developmental reorganization of fibers, cells, and extra-cellular matrix (ECM) in fetal and infant brain, we can reconstruct location of different classes of afferent axonal pathways within compartments of the cerebral wall (3, 8, 10, 15, 16, 23, 65–67). We found that deep, periventricular segments contain identifiable classes of axonal pathways (65). Using similar "segmental" topographical approach, we delineated axonal pathways in more superficial compartments of the cerebral wall (66). We propose that transient cerebral compartments serve as important spatial corridors for growth of different classes of axonal pathways. Therefore, we designate these transient cerebral compartments as "corridors of axonal growth."

The objectives of the present study were: (1) to define anatomical and developmental relationships between cerebral compartments and major axonal pathways and (2) to use this data for study of laminar location and extent of structural cerebral lesions in preterm infants at birth and during early postnatal life. We rely on structural criteria and parameters developed during our long-term study of normal and damaged cortex (54, 66).

The specific aims of this study are: (a) to define anatomically periventricular, intermediate, and superficial cerebral compartments, to identify incorporated classes of axonal pathways and to describe laminar landmarks for typical lesions in the preterm brain (question: where in the cerebral wall?); (b) to show the extent and characteristics of MR signal abnormalities in different cortical compartments and white matter segments at birth and in the subsequent longitudinal MR structural follow-up until the third year of life (question: how do cerebral compartments develop after lesion?); (c) to elucidate whether there are differences in structural abnormalities after the lesion in early versus late preterms, with special consideration of the subplate zone (question: when?).

The idea behind this approach is to determine whether analysis of structural abnormalities of laminar compartments and white matter segments along radial axis (from ventricle to pia) may reveal selective time-dependent and laminar-dependent radial vulnerability of the different classes of axonal pathways preterm brain (question: which pathways are lesioned in the white matter injury?). We expect that our findings will contribute to better classification and scoring of white matter injuries in preterm infant.

MATERIALS AND METHODS

For histological delineation of cerebral compartments and white matter segments, we used different fibrillar, cellular, and ECM markers on post-mortem human brains (age range 22–44 PCW) from our large and versatile Zagreb collection. For the analysis of the specimens with pathological changes, we have used same techniques as applied for normal brains in our previous studies. The details on histological, histochemical, and immunocytochemical techniques as well as selections of antibodies were described in details in our previous papers (65, 66). *In vivo* MRI examination was conducted using a set of MRI sequences, as described previously (65, 66) on three groups of children (**Table 1**).

The first group, consisting of 21 patients (age range at birth: 23–42 PCW), was selected from a cohort of 152 children included in another longitudinal study. The exclusion criteria were: the presence of developmental anomalies, higher-grade hydrocephalus, and massive infarctions. The inclusion criteria were: the presence of other structural lesion (visible on MRI scans) related to perinatal pathology. The severity of these lesions was graded according to the surveillance of cerebral palsy in Europe (SCPE) classification system (68, 69) as follows: non-cystic periventricular leukomalacia (PVL) (two unilateral and eight bilateral cases); cystic PVL (two unilateral and two bilateral cases); intraventricular or periventricular hemorrhage (one unilateral and four bilateral cases); and two patients with moderate basal ganglia/thalamus and cortex lesions (**Table 1**). All children in this group also had neurological disorders of different levels as revealed on clinical exams and SNAPII/SNAPPEII scores (70). The second group included 11 prematurely born babies (age range: 24–31 PCW), who had

Table 1 | Groups of children included in histological (*in vitro*) and MRI (*in vivo*) study with classification of lesion on MRI according to SCPE grading system.

Groups	Methods	Type of lesion	Number of cases		MRI1	MRI2
			Unilateral	Bilateral		
Premature and term children with pathology	*In vivo* MRI (23–42 PCW)	Non-cystic periventricular leukomalacia (27–30 PCW)	2	8	+	+
		Cystic periventricular leukomalacia (27–33 PCW)	2	2	+	+
		Intraventricular/periventricular hemorrhage (23–39 PCW)	1	4	+	+
		Moderate basal ganglia/thalamus/cortex lesion (40–42 PCW)	0	2	+	+
	In vitro histology (22–44 PCW)		7			
Normotypic prematures	*In vivo* MRI (24–31 PCW)		11		+	+
	In vitro histology (26–34 PCW)		12			
Normal term children	*In vivo* MRI (39–41 PCW)		4		+	−
	In vitro histology (39–41 PCW)		2			

Number in parenthesis refers to PCW age range at birth. First MRI exam (MRI1) was performed at term or term equivalent age and the second MRI (MRI2) exam was performed during period between first and third years.

no signs of neurological disorders and had normal brain morphology, as independently assessed by two neuroradiologists. This group of children was regarded as "normotypic." Both groups of prematurely born children underwent longitudinal MRI exams, the first at the term equivalent age, and the second during the period between the first and third postnatal year.

The third group was composed of four normal children born at term, who were scanned during the neonatal period (due to the extracranial indication), and had neither notable brain pathology nor any signs of neurological disorders. This group of newborns was regarded as normal. In each case, the parental consent for MRI scanning was obtained and all examinations were controlled and approved by the Institutional Review Board of the University of Zagreb School of Medicine. Sampling of the tissue for the *in vitro* experiments was performed in accordance with the Declaration of Helsinki and also was approved by the Institutional Review Board of the University of Zagreb School of Medicine. All MRI data were evaluated by two independent observers (Milan Radoš and Ivica Kostović), while histological sections were analyzed by the first author. Clinical data and testing were provided by neuropediatrician and psychologist.

For delineation of transient cerebral compartments, we use generally accepted classification (9) while the classification of crossroads and other white matter segments is used as defined previously (11, 18, 66).

Previously described MRI abnormalities at term (changes in signal intensity, loss or enhancement of borders between compartments, cysts, patchy hyperintensities, scars, periventricular hemorrhages, atrophy of white matter segments, ventriculomegalia) (11, 18, 30, 31, 35, 42, 53, 63, 64, 66, 71–73) were precisely located in one of cerebral compartments described in our previous study (66). For the purpose of the present study, we have divided cerebral compartments in to deep (periventricular), intermediate, and distal (superficial) compartments (see **Table 2**).

The analysis of histological sections stained with histochemical (AChE histochemistry) and immunohistochemical methods (fibrillar staining) and structural post-mortem MR images revealed that these compartments are arranged in radial direction from ventricular (deep) to the pial surface (superficial) (9, 54).

1. Deep (periventricular) compartment includes proliferative fetal zones (ventricular–subventricular zone and ganglionic eminence) and adjacent periventricular fiber systems (65). This compartment roughly corresponds to white matter segment I of Von Monakow (11, 18) (**Figure 1**). Periventricular compartment contains massive callosal system and periventricular fiber system (segment I), consisting of associative fronto-occipital fascicle (FOF), cortico-striatal fibers within the subcallosal bundle and fronto-pontine pathways (8) (see **Table 2**).
2. Intermediate compartment (intermediate zone of mid-fetal period) contains crossroads of projection pathways (segment IIa) (11), their growth trajectories within sagittal axon strata (segment IIb) (8, 18) and centrum semiovale (segment III), which develops in late preterms (**Figures 1A,D,G**). Intermediate compartment contains major main telencephalic fiber systems (12, 25, 26, 54, 74). Crossroads are composed of massive projection fibers in the root of corona radiata (with thalamocortical and cortico-fugal radiating fibers), which are crossed by

Table 2 | Compartmental organization of the brain with related axonal pathways.

Deep (periventricular) compartment	Corpus callosum – *(segment I)*
	Fronto-occipital fascicle (FOF) – (*segment I*)
	Cortico-striatal fibers (Muratoff's fascicle) – (*segment I*)
	Fronto-pontine pathways – (*segment I*)
Intermediate compartment	Crossroads of projection pathways – (*segment II*)
	– Thalamocortical fibers
	– Cortico-fugal fibers
	– Callosal radiation
	– Associative sagittal fibers
	Sagittal axonal strata – (*segment II*)
	– Thalamocortical pathways
	– Basal forebrain cholinergic afferents
	– Cortico-cortical associative fiber system
	Centrum semiovale – (*segment III*)
	– Long associative fiber system
	– Projection fibers
Superficial compartment	Gyral white matter – (*segment IV*)
	– Short cortico-cortical fibers
	– U-fibers
	Subplate/subplate remnant
	– Growing front of all afferent pathways
	– Short cortico-cortical fibers
	Intracortical fibers – (*segment V*)

Axonal pathways are divided in segments (I–V) according to Von Monakow. Table is complementary to ***Figure 6****.*

callosal radiation and the deepest associative sagittal fibers, surrounded by large amount of ECM (11). Sagittal strata (segment IIb) are most prominent in the occipital lobe and contain projections from sensory thalamus, projection from associative thalamus (pulvinar) and capsula externa radiation with basal forebrain cholinergic afferents and cortico-cortical associative fibers (15, 16). The centrum semiovale (segment III) (75) is composed of massive long associative fiber systems and projection fibers (see **Table 2**).

3. Superficial compartment is composed of three transient zones constituting the neocortical anlage: the subplate, the cortical plate, and the marginal zone (**Figures 1D,G**). During the late gestation and perinatal period, the gyral white matter (segment IV) develops in the superficial compartment. The subplate is fibrillar, deep portion of the cortical anlage, containing different cellular elements: postmigratory neurons with early functional activity, early formed synapses, axonal plexus of "waiting" afferent fibers, migratory neurons, different glial cell lines, and large amount of ECM (23). MRI properties of the subplate (high intensity on T2 and low intensity on T1 sequences) are mainly caused by large extra-cellular space, hydrophilic ECM, and anisotropic structure. The gyral white matter (segment IV) and intracortical fibers (segment V) are poorly developed in preterm brain. Superficial compartment is dominated by the subplate zone [not defined by Von Monakow; for delineation criteria see Ref. (54)]. Before 26 PCW, the subplate contains "waiting" afferent fibers from thalamus arranged in fibrillar network, and in later preterm period (after 28 PCW) growing front of the most superficial associative fibers. The gyral white matter (segment IV) develops during the late gestation and parallel with resolution of the subplate becomes closely adjacent to the cortical plate. However, in the neonatal brain, the subplate remnant still exists and serves as a growth zone for short cortico-cortical fibers and U-fibers (66).

The main differences between the cerebral wall of late versus early preterms were described recently (66). For the purpose of this study, it is important to note the following differences: an enlargement of the centrum semiovale, the formation of sulci with reduction of the subplate (**Figure 1G**), and thickening of the corpus callosum (callosal plate).

RESULTS

Using the above described spatial (topographical) and temporal (developmental age) criteria, we will describe: (a) typical lesions and their laminar landmarks in preterm infants, (b) morphological types and radial extent of MR abnormalities in preterms at term age, and (c) structural longitudinal *in vivo* MRI changes after the first year of life in the same group of patients who were scanned at term age.

TYPICAL LESIONS IN PRETERM INFANTS AND ITS LAMINAR LANDMARKS

Lesions in the periventricular compartment – periventricular pathway zone

Periventricular lesions occupy area medial to the radiation of internal capsule (**Figure 1**, dotted line). Two types of lesion were seen to be restricted to the zone of periventricular pathways. The first is acute, localized periventricular hemorrhage in the space between the ganglionic eminence and the periventricular pathway zone (PVP) zone (**Figure 1B**). The ganglionic eminence is the most prominent, cell-dense periventricular structure in the preterm brain (**Figures 1A,B**). The PVP pathway zone is triangular area situated at the lateral angle of lateral ventricle (**Figures 1A,D**). The second type of lesion is cystic formation (with cavity) situated in the PVP pathway zone (segment I). Larger hemorrhagic lesions extend to the exit of the internal capsule (**Figure 1C**). This type of lesion affects all periventricular pathways [the subcallosal fascicle with cortico-striatal fibers, the fronto-pontine motor pathway, and the most massive FOF (65)] and extends into the intermediate compartment (segment II, see **Table 2**).

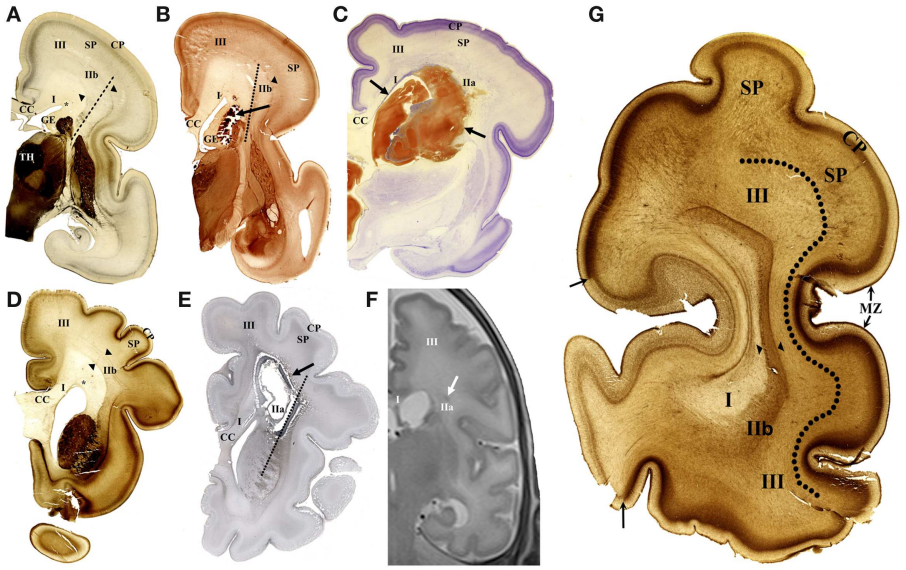

FIGURE 1 | Cellular compartments in the cerebral wall of the preterm brain and typical lesions shown on coronal sections. The acetylcholinesterase (AChE) histochemistry **(A,B,D,G)**, Nissl-stained section **(C)**, immunohistochemical fibrillar staining **(E)**, and *in vivo* MR imaging **(F)** in early preterm of 26 PCW **(A–C)** and late preterm of 35 PCW **(D–G)**. In early preterm, deep periventricular compartment consists of ventricular–subventricular zone, ganglionic eminence (GE), and white matter segment (WMS I). Intermediate compartment contains crossroads (WMS IIa), sagittal strata (WMS IIb – between arrowheads), and centrum semiovale (WMS III). Typical small hemorrhagic lesion is visible in periventricular segment I **(B)**. Large hemorrhagic lesion [between arrows in **(C)**] destroys WMS I and IIa. In late preterm, cystic lesion is shown extending throughout WMS I and IIa **(E)**. Small hemorrhagic lesion is located in WMS IIa on *in vivo* MR image **(F)**. AChE histochemistry shows main compartments and WM segments on coronal section through occipital lobe **(G)**. Periventricular compartment, including WMS I, is followed in radial direction with sagittal strata (WMS IIb, between arrowheads). Next compartment, centrum semiovale (III) is delineated from subplate (SP) with broken line. Arrows indicate border between primary visual cortex (light staining) and area 18 (heavy staining). TH, thalamus; SP, subplate; CC, corpus callosum; MZ, marginal zone; asterisk, PVP pathway zone; broken lines **(A,B,E)** indicate axis of internal capsule.

Lesions of intermediate compartment

Larger cystic lesions (**Figure 1E**) also involve the segment II that is periventricular crossroads of pathways (11). The presence of thick fibrillar–glial capsule around the cystic lesion indicated early development of this lesion. MR imaging offers a unique opportunity to visualize small lesions in the periventricular crossroads (**Figure 1F**).

We conclude that these periventricular focal lesions in preterm infant damage segment I of white matter (PVP) and segment IIa (crossroads) and cause MR abnormalities, leaving intact the subplate zone/centrum semiovale and gyral white matter (in later preterms). Lesions of the occipital crossroads are accompanied with lesions of the root of radiation of the posterior limb of the internal capsule (PLIC) and may also affect occipital sagittal strata. The lesions of periventricular and crossroad segments also damage proliferative and migratory zones within the cerebral wall.

As stated above, the centrum semiovale is situated between sagittal strata and the subplate, and was developed only in later preterms (**Figure 1G**). The most common finding ("abnormality") is an increase in MRI T2 signal intensity (73). This developmental abnormality corresponds topographically to the definition of diffuse periventricular leukomalacia (31).

We found that decrease in visibility of anatomical border between sagittal strata and centrum semiovale – subplate, may be more indicative of the prospective acute lesion of centrum semiovale than the change in MRI signal intensity alone (**Figure 1F**). The external capsule radiation is the only reliable landmark (54) for anatomical delineation between focal periventricular lesions and diffuse lesions in the centrum semiovale (73). These landmarks can be easily determined only in the frontal and occipital lobe (**Figure 1G**).

Lesions of superficial compartment

In this study, lesions of the subplate and the cortical plate have not been described convincingly at MRI level. However, at the histological level, there is evidence for significant reactivity of astroglia in the deep portion of subplate after hypoxic-ischemic lesions (76) and widespread (although non-specific) changes of subplate neurons (52).

MORPHOLOGICAL TYPES AND RADIAL EXTENT OF MRI ABNORMALITIES IN PRETERM CHILDREN AT TERM AGE IN REFERENCE TO WHITE MATTER SEGMENTS AND THE SUBPLATE REMNANT

The neonatal brain at term age shows well developed deep and intermediate segments of white matter (segments I, II, and III), while distal segment (gyral white matter) is still developing. The subplate is reduced in thickness and is described as the subplate remnant (66). The neocortex is fully laminated, but appears very immature due to the higher packing density of its neurons. The

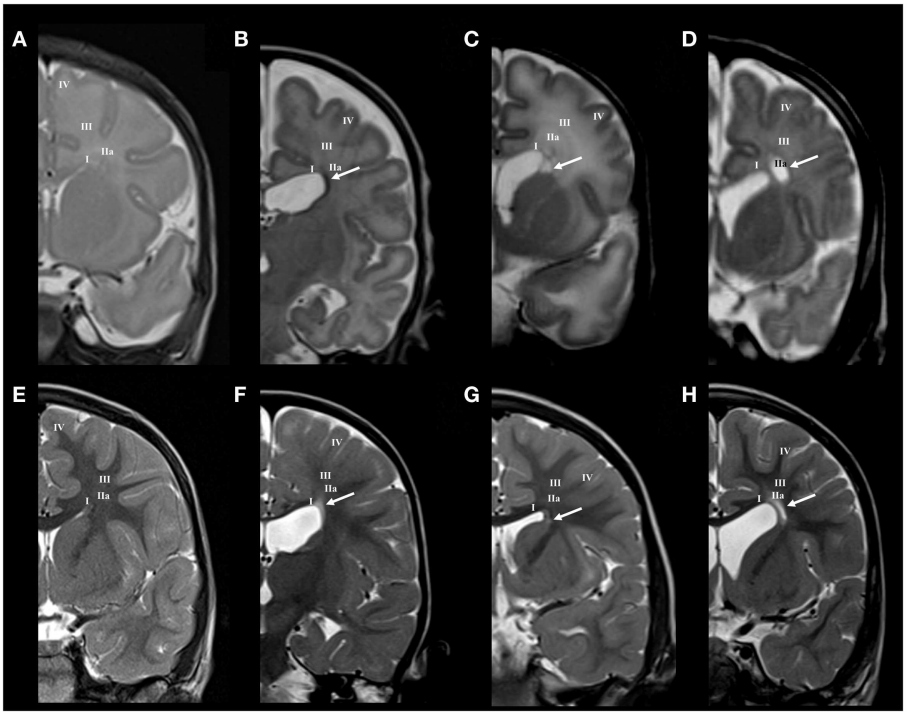

FIGURE 2 | Longitudinal MRI follow-up of perinatal periventricular pathways lesions on coronal T2 images. Normal findings of periventricular white matter at term **(A)** and at the age of 3 years **(E)**. Early preterm [born at 25 PCW, birth weight (BW) = 800 g, SNAPII = 35, SNAPPEII = 63, Apgar score (AS) = 9/10] with hemorrhagic lesion in area of subcallosal fascicle at term equivalent age [arrow in **(B)**], which changed to gliotic scar at the age of 2 years [arrow in **(F)**]. Early preterm (born at 28 PCW, BW = 1080 g, SNAPII = 13, SNAPPEII = 31, AS = 2/4) with cystic lesion in area of subcallosal and fronto-occipital fascicle at term equivalent age [arrow in **(C)**], which changed to discrete scar within fronto-occipital fascicle at the age of 3 years [arrow in **(G)**]. Late preterm (born at 31 PCW, BW = 1670 g, SNAPII = 8, SNAPPEII = 8, AS = 8/10) with large cystic lesion at the crossroad area at term equivalent age [arrow in **(D)**], which appears as smaller cystic lesion at the age of 3 years [arrow in **(H)**]. Numbers I–IV represent segments of white matter as previously described in text.

white matter segment V (radii), which consist of bundles of radially arranged axons, is poorly developed. In preterm infants at term, the crossroads, centrum semiovale and developing gyral white matter show, in higher percentage than normal term brain, an increase in MRI signal intensity (**Figure 2**).

Lesion of periventricular compartment

Small hemorrhagic lesions (five cases) were seen to occupy territory of Muratoff's subcallosal fascicle in the PVP, lateral to the angle of the ventricle (**Figure 2B**). Periventricular cystic lesions (four cases) destroy, in addition to subcallosal fascicle, more laterally positioned FOF (**Figure 2C**).

Lesion of intermediate compartment

Larger periventricular cysts stretch to the exit of internal capsule/root of corona radiata (**Figure 2D**) and involve crossroads of pathways (segment IIa). Lesions in the territory of sagittal strata are characterized by decrease in the visibility of borders between individual sagittal strata and the border between external sagittal stratum and centrum semiovale (**Figure 3C**). The most common MRI abnormality of centrum semiovale is an increase in T2 MRI signal intensity (**Figure 4B**). The characteristic abnormality is decrease in visibility of border between intermediate compartment (centrum semiovale and sagittal strata) and superficial compartment (gyral white matter and subplate remnant). This abnormality is prospective MRI structural evolution of the so-called diffused PVL.

Lesion of the superficial compartment

The presence of the subplate remnant, defined as narrow ECM rich transitional zone between the gyral white matter and the cortical plate, is marker of normal cortical organization at birth (66). Accordingly, the absence of delineation of the subplate remnant or sharp (enhanced) delineation (**Figure 4**) is a marker of possible lesion of distal (superficial) compartment of the cerebral wall during the late preterm period. The close spatial and developmental relationships between the subplate remnant and underlying gyral white matter make MRI delineation of these two sub-compartments extremely difficult. The presence of these transient sub-compartments may be demonstrated using different cellular, extra-cellular, and fibrillar markers at fine histological level (66). However, if there are significant regional changes in MRI signal intensity of the gyral white matter (**Figure 5B**), with loss of borders, one should consider the existence of abnormality of these two superficial sub-compartments. This can be confirmed by longitudinal imaging after the first year of life.

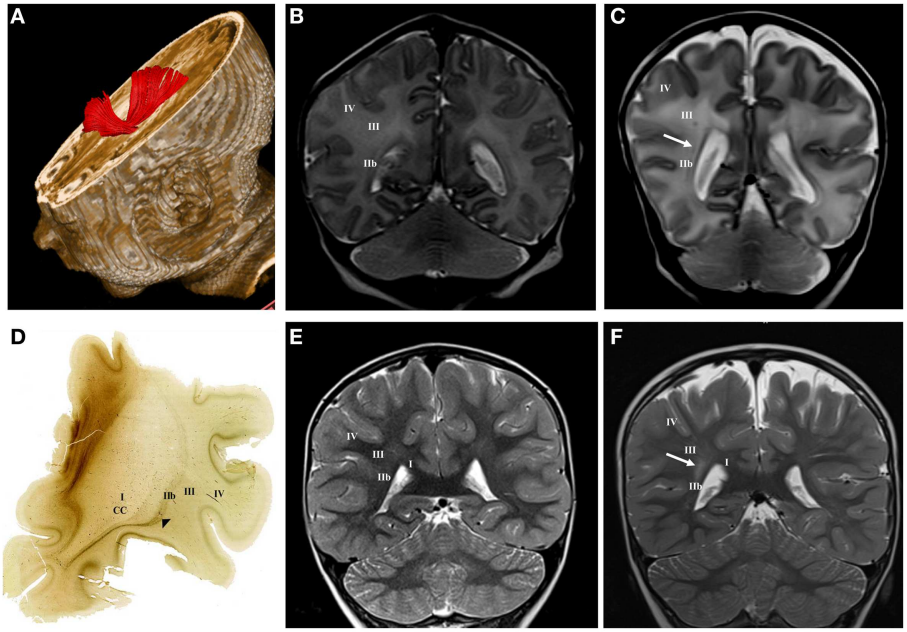

FIGURE 3 | DTI tractography reconstruction of sagittal strata at term age (A). The acetylcholinesterase (AChE) histochemistry shows external sagittal stratum [arrowhead in **(D)**] at the frontal level at term age. Normal MRI findings of sagittal strata at term age **(B)** and at the age of 3 years **(E)** on T2 coronal images. Late preterm child (born at 30 PCW, BW = 1510 g, SNAPII = 16, SNAPPEII = 34, AS = 2/5) with punctate hemorrhagic lesion within sagittal strata at term equivalent age [arrow in **(C)**] and hyper-intensive lesion of sagittal strata at the age of 3 years [arrow in **(F)**] on T2 coronal images. Numbers I–IV represent segments of white matter as previously described, CC, corpus callosum.

STRUCTURAL (LONGITUDINAL) *IN VIVO* MR CHANGES OF WHITE MATTER AND CELLULAR COMPARTMENTS AFTER THE FIRST YEAR OF LIFE

The longitudinal developmental changes of cerebral compartments were analyzed in the same cohort of patients, which was described at term age.

Periventricular compartment

As expected, small hemorrhagic lesions in PVP area transformed in focal well delineated "scar-like" formation of increased T2 MRI signal intensity (**Figure 2F**). Small cystic lesions of periventricular compartment disappear. Instead, the area of periventricular pathways shows mild shrinkage with slight reduction of FOF (**Figure 2G**). Abnormalities of signal intensity and "scars" in the periventricular compartment may be confluent along the entire dorsal ventricular system.

Intermediate compartment

Large cystic lesions collapsed, but remained visible in the territory of crossroads and periventricular zone (**Figure 2H**). Semi-oval cystic formation may extend along the entire cerebral wall. After the first year, the distal portion of intermediate compartment (segment II), that is sagittal strata, shows loss of characteristic three-band appearance with abnormal signal elongated in the sagittal plane (**Figure 3F**). Abnormalities of MRI signal intensity, observed in PLIC area at term, later show narrower distance between sides of triangular crossroad area at the point where external and internal capsule continue in external and internal sagittal stratum. The most interesting finding in the intermediate compartment and distal compartment is enhanced difference between MRI signal intensity of centrum semiovale and subplate remnant (**Figure 4D**).

Superficial compartment

In cases with regional gyral white matter lesions, there is selective MRI signal abnormality with loss of border with adjacent compartments. In these cases, the centrum semiovale shows normal MRI signal intensity, which indicates a proper myelination process (**Figure 5**).

In conclusion, the analysis of cerebral compartments and white matter segments after the first year shows, in some cases, that an increased MRI T2 signal intensity observed at term did develop in a characteristic MRI abnormality: the sharp delineation of the subplate remnant and U-fiber zone and MRI signal abnormalities remain present in the centrum semiovale. This indicated selective vulnerability of main body of associative fibers in the centrum semiovale.

DISCUSSION

We have reviewed the evidence on organization and developmental dynamics of cellular compartments in the cerebral wall in the third trimester of human gestation and illustrated how precise anatomical landmarks can be used for description of radial extent of lesion on histological sections and conventional MR images. In addition, we evaluated the significance of borders between cerebral compartments for MRI analysis of abnormalities of premature infant brain at term equivalent age and documented their structural reorganization in longitudinal (second MR) imaging. The concept of transient cellular compartments as a crucial spatial

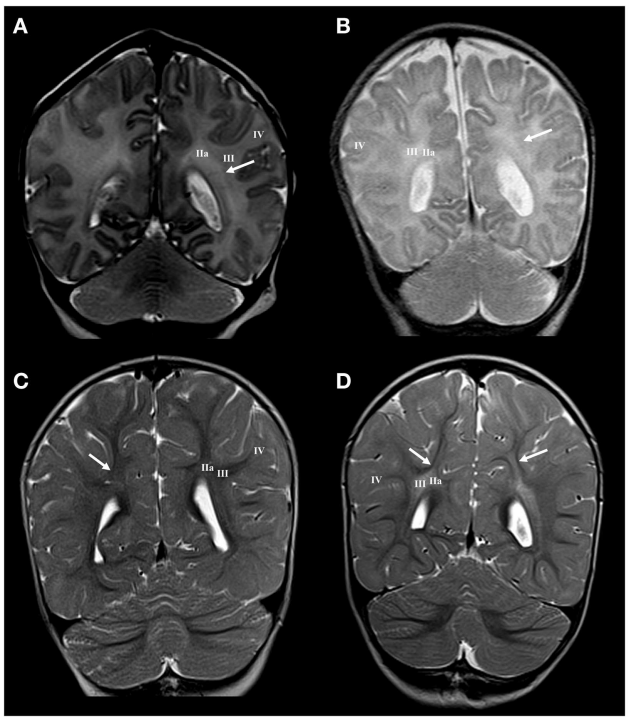

FIGURE 4 | Longitudinal MRI follow-up of centrum semiovale perinatal lesion on coronal T2 images. Normal findings of centrum semiovale at term age **(A)** with visible border between parietal crossroad and centrum semiovale [arrow in **(A)**]. Normal findings at the age of 13 months with barely visible border between U-fibers and centrum semiovale [arrow in **(C)**]. Term born child (BW = 3960 g, SNAPII = 40, SNAPPEII = 58, AS = 0/4) with perinatal asphyxia and diffuse hyperintensity of white matter with diminished border between parietal crossroad and centrum semiovale at term equivalent age [arrow in **(B)**] but with enhanced visibility of border between U-fibers and hyper-intensive centrum semiovale at the age of 13 months [arrows in **(D)**]. Numbers I–IV represent segments of white matter as previously described.

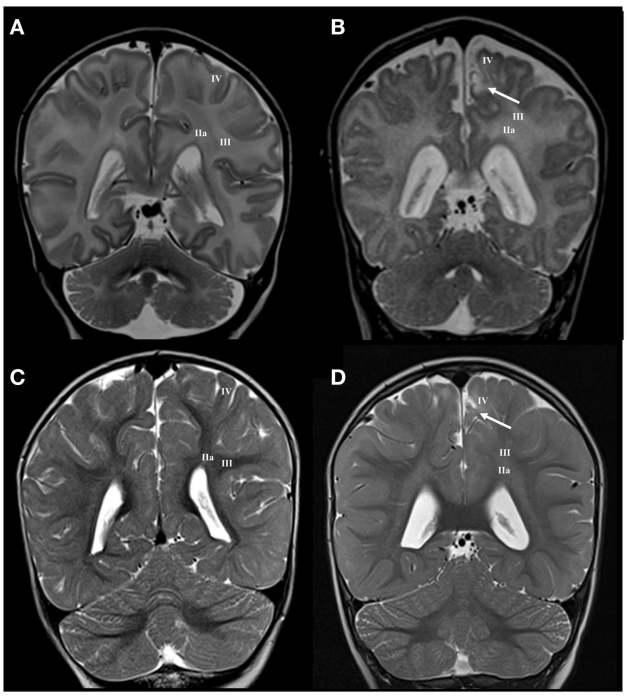

FIGURE 5 | Longitudinal MRI follow-up of perinatal gyral white matter and cerebral cortex lesion on coronal T2 images. Normal findings of gyral white matter and cortex at term age **(A)** and at the age of 13 months **(C)**. Term born child (BW = 3220 g, SNAPII = 31, SNAPEII = 49, AS = 1/3) with perinatal asphyxia and hyperintensity of centrum semiovale and gyral white matter accompanied with cortical lesion at term equivalent age [arrow in **(B)**] and with hyper-intensive lesion of gyral white matter and cortex at the age of 13 months [arrow in **(D)**]. Numbers I–IV represent segments of white matter as previously described.

framework for analysis of neurogenetic events in the developing cerebral cortex has been elaborated for decades since the beginning of modern era of developmental neurobiology (9, 13, 19, 22). There is a general agreement that dynamic changes of transient compartments reflect basic pattern of histogenesis of the developing cerebral cortex (9, 19, 54, 77–79). Laminar organization of fetal cerebral compartments was useful for description of developmental changes of the cerebral cortex in current imaging studies (12, 24–26, 28, 54, 66, 80).

Due to the fact that neurogenetic events take place in specific, developmentally important cerebral compartments, the laminar extent of cerebral lesion may help to understand developmental disturbances after hypoxia ischemia and hemorrhagic lesion in the third trimester of gestation and equivalent preterm period (30, 34, 53, 62). In the present paper, we have extended the conceptual framework of transient compartments to the concept of radial vulnerability of different white matter segments and compartments in the cerebral wall. The distribution of different classes of well-known cortical projection, commissural and associative pathways arranged radially (from ventricle to pia) within different spatial compartments, may be easily related to classical description of focal periventricular and diffused lesions in preterm brain (30, 31, 53, 61, 63, 64).

We found that both focal and diffused lesions, depending on their radial extent, affect well identified and spatially segregated classes of axons: periventricular pathways of mixed modalities within ventricular/subventricular zone (65), major projection pathways within the crossroads (11, 54) and sagittal strata (18, 54, 75, 81), associative pathways within the centrum semiovale and deep cortical subplate and thalamocorticals in the subplate of early preterms. Moreover, characteristic spatial arrangement of these axonal pathways shows time-related sequential growth, with some overlap during 22–36 PCW. These two parameters, spatial (compartments) and developmental (periods), are two factors, which determine the extent and nature of lesions of white matter pathways, the subplate and incorporated nerve cells (31, 82), resulting in dynamic picture of vulnerability across the cerebral wall. By analyzing MR images in small cohort of premature infants at birth and after the first year of life, as well as histological sections of selected post-mortem cases, we found that there are well delineated lesions, which almost selectively damage early

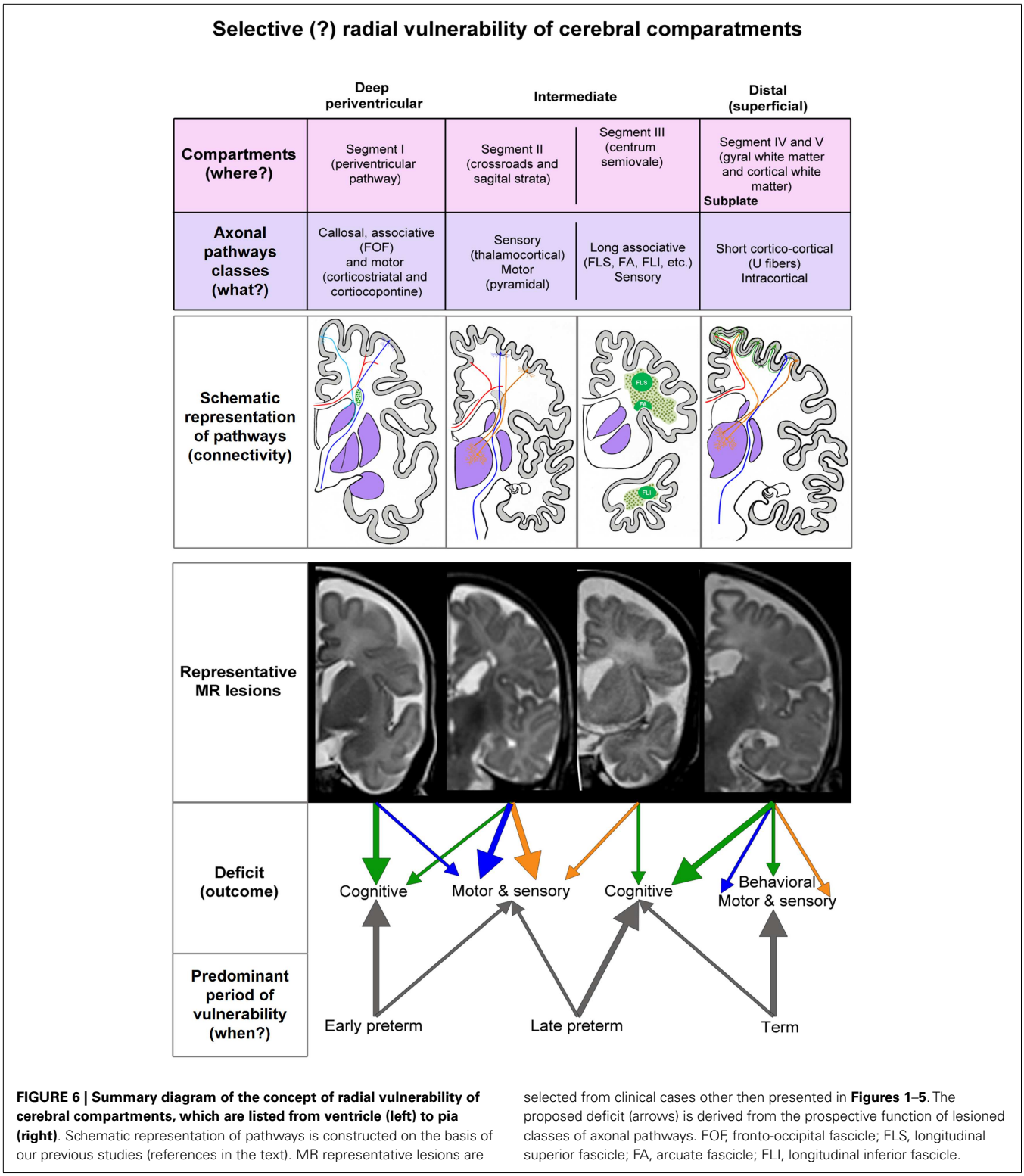

FIGURE 6 | Summary diagram of the concept of radial vulnerability of cerebral compartments, which are listed from ventricle (left) to pia (right). Schematic representation of pathways is constructed on the basis of our previous studies (references in the text). MR representative lesions are selected from clinical cases other then presented in **Figures 1–5**. The proposed deficit (arrows) is derived from the prospective function of lesioned classes of axonal pathways. FOF, fronto-occipital fascicle; FLS, longitudinal superior fascicle; FA, arcuate fascicle; FLI, longitudinal inferior fascicle.

differentiating associative FOF and motor pathways in the periventricular zone situated medial to the root of the corona radiata. This periventricular focal lesion may be easily followed from term equivalent age to the second MRI scan after the first year of life. The prospective deficit after this type of lesion is not well defined, but it may include general cognition due to the damage of the robust FOF and impairment of motor functions related to the damage of fronto-pontine and cortico-striatal pathways (**Figure 6**). The lesions in this medial periventricular area are complicated by the fact that the very same lesion may damage cell proliferation in

the ganglionic eminence and migration of GABAergic neurons from the ganglionic eminence (8). The most complex and difficult to interpret may be lesions of periventricular crossroads of pathways (11). Kidokoro and Inder (64) have shown that visibility of crossroads is a sign of normal development while poor delineation combined with increase in MRI signal intensity is related to poor neurodevelopmental outcome. We confirmed this finding, but also extend this observation to the sagittal strata: we demonstrated that decrease in visibility and delineation of three sagittal strata (internal, central, and external) (8) in the frontal and occipital lobe are important signs of MRI abnormality. These MRI abnormalities probably indicate the lesions of thalamocortical sensory pathways and pathways from the associative thalamic nuclei, such as pulvinar, which are the most voluminous contingent of intermediate sagittal stratum (18, 54). In our previous studies, we emphasized that during the period between 24 and 26 PCW thalamic fibers from mediodorsal nucleus and pulvinar complex accumulate below the cortical plate and penetrate frontal and parietal associative cortex (3, 15, 16, 82).

Thus, loss of "normal" border between external sagittal stratum and centrum semiovale is indicative of lesion of thalamocortical pathways and may be accompanied with thalamic lesions (31, 42, 73, 83, 84).

One of the most interesting observations in our material is lesioning of centrum semiovale with preservation of the subplate remnant. This developmental lesion results in loss of border at term, but shows "enhanced" delineation of subplate remnant from centrum semiovale (segment III) during the subsequent development. This reorganization of borders between cerebral compartments requires developmental interpretation of subplate zone, centrum semiovale, related thalamocortical and associative pathways.

The centrum semiovale develops in the late preterm, between 32 and 36 PCW, when thalamocortical fibers have already relocated from the subplate zone (4, 10, 19). The centrum semiovale and the deep subplate serve as main compartments for growth of long associative cortico-cortical fibers. Some of these growing pathways may be damaged by diffuse distal hypoxic ischemic lesions (31). As a consequence, centrum semiovale shows signal abnormality at birth. After the first year, impairment of myelination and other unexplored pathological (developmental?) changes may cause abnormal MRI signal. On the other hand, short cortico-cortical fibers (including U-fibers) grow through the subplate remnant during a different developmental period that is during the early postnatal life. Therefore, they are not damaged during the preterm period (like long cortico-cortical fibers) and show "normal" MRI signal intensity during the postnatal period. Thus, we interpret the phenomenon of enhanced border between the subplate remnant and the centrum semiovale as a consequence of differential period of vulnerability for these two compartments.

The lesion of subplate neurons is the most enigmatic. There is no doubt that subplate neurons are vulnerable during preterm period and form a neuronal substrate in Volpe's "complex amalgam of destructive and developmental disturbances." This prediction was confirmed in neuropathological studies after hypoxic ischemic lesion (52). Considering complex developmental role of subplate zone and subplate neurons (10, 23, 85–87) damage of subplate neurons may have multiple effects on cortical development. From developmental studies (23, 88), it can be predicted that lesion of subplate neurons and other subplate cellular and extra-cellular elements will interfere with growth of thalamocortical fibers. As stated above, we believe that the growth-related vulnerability of associative cortico-cortical pathways in this distal segment of white matter (centrum semiovale) at the interface with subplate occurs later then vulnerability of deeper thalamocortical fibers containing segments of fetal white matter (sagittal strata and crossroads including PLIC). If our prediction is correct, then vulnerability of thalamocortical projection axonal pathways occurs between 22 and 28 PCW that is in early preterm period, when these fibers wait and accumulate in subplate and penetrate cortical plate.

The mechanisms that contribute to the pathology of axonal pathways in preterm brain are poorly understood. It is also not clear whether, when and how growing axons are primary target for hypoxia ischemia or their lesion occurs secondary to the damage of their cell bodies of origin, other connected neurons, such as subplate neurons or glia cells (31, 53).

Haynes et al. (89) have shown that damage of axons may be significant component of diffuse PVL and can be detected by the apoptotic marker fractine. The axons may lose their integrity because they fail to find their path due to the hypoxic ischemic disturbance of ECM or death of cells, which secrete axonal guidance molecules. The axonal guidance is event with complex interactions of receptor molecules and chemo-attractant or chemo-repellant molecules, which are present and expressed in characteristic gradients within the cerebral wall (5, 90).

This complex axonal guidance system is the main mechanism in the complex process of path-finding and target selection (2, 5–7, 19). The distribution of large amount of ECM in vulnerable compartments such as periventricular crossroads and subplate, lead us to propose that one of the most important basic mechanisms in periventricular white matter injury and encephalopathy of prematurity (53) is abnormality in organization and content of ECM and impaired synthesis of axonal guidance molecules (11, 66).

Although it is not known which cells produce ECM molecules such as chondroitin sulfate, containing glycosaminoglycans, it is very likely that these cells reside along axonal growth trajectories and in the subplate. It is logical to assume that cytotoxic substances produced during hypoxic ischemic events damage not only pre-oligodendrocytes (53, 91) but also affect astrocytes (31, 76). Astrocytes are distributed within all compartments of cerebral wall during the third trimester of gestation and are candidates for different metabolic and synaptic functions, including the synthesis of ECM. It is interesting that astrocyte activation is more characteristic of late preterm period (76) and also involves subplate astroglia without macroscopic changes (31, 76, 92). Recent studies of white matter injury in preterm brain suggest that damage of pre-oligodendrocytes place important role in the pathogenesis of prenatal hypoxia (48), especially in the diffuse component of periventricular leukomalacia (93). This view is consistent with opinion that impaired myelination is important factor in axonal deficit and contributes to the decrease in white matter volume in prematurely born infants (46). Since centrum semiovale with massive associative pathways is involved in diffuse non-cystic white

matter injury, hypoxic ischemic lesions of pre-oligodendrocytes may contribute to the changes observed in this compartment in our material. Immunocytochemical preparations for myelin basic protein (MBP) of post-mortem brains of children who died with evidence of hypoxic-ischemic episodes show preserved myelination of projection fibers but poor myelination of centrum semiovale (65). This corresponds to our MRI findings. One of the most important conclusions from the present paper is that the time of injury and radial extent of lesion from ventricle to pia have effects on subsequent organization of white matter and cortex. This is in agreement with the concept that encephalopathy of prematurity is an amalgam of destructive and developmental disturbances (31). Our data relevant to this concept show that, when imaging and histological data are presented for all compartments and segments of white matter, from ventricle to pia (66), and for borders between compartments and changes in MRI signal intensity (63, 64, 72) clear spatial relationships with histological landmarks can be detected (54, 66).

The concept of radial vulnerability and data presented in this paper are limited due to the fact that we did not analyze connected subcortical structures (caudate, putamen, thalamus, amygdala, cerebellum, brain stem nuclei, and spinal cord). This will be subject of our future studies.

CONCLUSION

In conclusion, developmental vulnerability changes along radial axis in relation to growing axonal strata and deep to superficial differentiation of neurons in the subplate and cortical plate. Deep, periventricular lesions (PVH and focal PVL) damage fronto-occipital associative, cortico-striatal, and corticopontine projection pathways and will result in cognitive and motor deficit (**Figure 6**). Periventricular lesions also interfere with proliferation and migration, which contributes to the complexity of the lesion. Lesions within the internal capsule, crossroads, and sagittal strata damage predominantly projection pathways (sensory and motor), with possible cognitive component. Vulnerability of thalamocortical pathways within the crossroad and sagittal strata seems to be characteristic for early preterms, while vulnerability of association pathways in the centrum semiovale seems to be predominant feature of late preterms. The cerebral compartments, which are not affected in the preterm brain, the superficial subplate, and the cortical plate with short cortico-cortical fibers, are important substrate for later developmental plasticity and functional recovery of the damaged infant brain. However, if damaged during prenatal period, the subplate zone, subplate cells, cortical cells, and short cortico-cortical connections will cause cortical type of deficit and combination of behavioral, motor, sensory, and cognitive components. The delineation between different, intermediate, and superficial segments, segments of white matter (external sagittal stratum, centrum semiovale, and gyral white matter) subplate remnant and changes in signal intensity together with radial extent of MRI abnormalities seem to be important indicators of lesions of association pathways in prematurely born infants at term. In contrast, short cortico-cortical and U-fibers seem to be intact due to the late developmental schedule. Our study revealed that analysis of radial extent and laminar delineation of MRI abnormalities in the cerebral compartments may indicate lesion of different classes of axonal pathways and may help in prediction of structural and functional outcome after prenatal and perinatal lesions.

AUTHOR CONTRIBUTIONS

All authors have participated equally in the work and have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Ivica Kostović has proposed basic concept of cerebral compartments, corridors of axonal growth, and radial vulnerability. Vesna Benjak and Mirna Kostović-Srzentić have performed clinical testing of preterm children and Nataša Jovanov-Milošević has performed histochemical and immunohistochemical staining of post-mortem brain. Milan Radoš has performed all *in vivo* MR scans. All authors (Ivica Kostović, Vesna Benjak, Mirna Kostović-Srzentić, Nataša Jovanov-Milošević, and Milan Radoš) have participated equally in interpretation of data, article writing, and approval of final version.

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Upper limb function and cortical organization in youth with unilateral cerebral palsy

Anna Mackey*[1], *Cathy Stinear*[2], *Susan Stott*[1] *and Winston D. Byblow*[3]

[1] *Department of Surgery, University of Auckland, Auckland, New Zealand*
[2] *Department of Medicine, Centre for Brain Research, University of Auckland, Auckland, New Zealand*
[3] *Department of Sport and Exercise Science, Centre for Brain Research, University of Auckland, Auckland, New Zealand*

Edited by:
Anna Purna Basu, Newcastle University, UK

Reviewed by:
Pratibha Singhi, Postgraduate Institute of Medical Education and Research, India
Brigitte Vollmer, University of Southampton, UK

***Correspondence:**
Winston D. Byblow, Department of Sport and Exercise Science, Centre for Brain Research, University of Auckland, Auckland, New Zealand
e-mail: w.byblow@auckland.ac.nz

Aim: To explore the relationship between motor cortical and descending motor pathway reorganization, lesion type, and upper limb function in youth with unilateral cerebral palsy (CP).

Methods: Twenty participants with unilateral CP (mean age 15 ± 3 years; 11 males) completed a range of upper limb functional measures. Structural MRI, diffusion-weighted, and functional MRI were conducted to determine type and extent of brain lesion, descending white matter integrity, and whole-brain activity during affected hand use. Single pulse transcranial magnetic stimulation (TMS) ($n = 12$) was used to examine functional integrity of the corticospinal pathway as well as primary motor cortex intracortical and interhemispheric inhibition from motor-evoked potentials and silent periods.

Results: Fractional anisotropy measures within the posterior limb of the internal capsule were a predictor of upper limb function ($R^2 = 0.41$, $F = 11.3$, $p = 0.004$). Participants with periventricular lesions tended to have better upper limb function [$F(2, 17) = 42.48$, $p < 0.0001$]. Five participants with evidence of cortical reorganization and functional ipsilateral projections to their affected hand had worse upper limb function. Deficits in intracortical and interhemispheric inhibitory mechanisms were found in participants with worse upper limb function (Melbourne Assessment of Unilateral Upper Limb Function: Mann Whitney $p = 0.02$).

Conclusion: Neuroimaging and TMS can provide useful information related to hand function of individuals with unilateral CP and may have potential to assist as a predictive tool and/or guide rehabilitation.

Keywords: cerebral palsy, cortical re-organization, upper limb function, hemiplegia

INTRODUCTION

Unilateral cerebral palsy (CP) occurs after an insult to the developing brain, resulting in motor and sensory impairments (1). The subsequent upper limb functional deficits can significantly impact on a child's independence and potentially limit future employment options. A recent systematic review of upper limb therapeutic interventions for children with unilateral CP found a variable effectiveness of current treatments, with a lack of information on how and when to best target interventions (2). This paucity of information is a potential barrier to rehabilitation because it is difficult to appropriately select individuals who are best suited for particular interventions, such as those that are time and labor-intensive, e.g., constraint-induced movement therapy (CIMT).

There is increasing interest in whether neuroimaging can be used to predict response to intervention or therapy in CP (2–4). The timing, size, and location of the lesion influence the topographical presentation of the limbs affected (5), but the determination of the functional outcome for the child is more difficult to predict from structural MRI alone (6). Reorganization of the primary motor cortex (M1) is often evident in unilateral CP. Functional MRI (fMRI) during motor tasks may indicate shifts in activation toward the unaffected hemisphere when using the affected upper limb (7, 8). Motor-evoked potentials (MEPs) from transcranial magnetic stimulation (TMS) of M1 may also reveal reorganization, such as an increased prevalence of MEPs from TMS of the M1 ipsilateral to the affected side (9). The functional consequences of this reorganization are not fully understood.

Abbreviations: BOLD, blood oxygenation level-dependent; C/SC, cortical and subcortical lesion; CIMT, constraint-induced movement therapy; CL, contralesional hemisphere opposite to lesion; CP, cerebral palsy; cSP, contralateral silent period; DW-MRI, diffusion-weighted MRI; ECR, extensor carpi radialis; EMG, electromyography; FA, fractional anisotropy; FAAI, fractional anisotropy asymmetry index; FDI, first dorsal interosseous; fMRI, functional MRI; IL, ipsilesional hemisphere on side of lesion; iSP, ipsilateral silent period; LI, laterality index; M1, primary motor cortex; MACS, manual ability classification system; MAL, malformations; MEP, motor-evoked potential; MRI, magnetic resonance imaging; MSO, muscle stimulator output; MUUL, Melbourne assessment of unilateral upper limb function; PLIC, posterior limb of internal capsule; PV, periventricular; RMT, resting motor threshold; ROI, region of interest; TMS, transcranial magnetic stimulation.

The aim of this study was to explore the relationship between upper limb function measures in unilateral CP and characteristics of reorganization within and between motor cortices. Anatomical and diffusion-weighted MRI (DW-MRI) was used to assess structural integrity of descending motor pathways, while TMS and fMRI were used to assess the functional integrity and organization of the corticomotor pathways.

MATERIALS AND METHODS

The study was approved by the New Zealand Health and Disability Ethics Committee, with written consent obtained from participants and parents, in accordance with the Declaration of Helsinki.

PARTICIPANTS

We recruited 20 youths from local orthopedic and therapy services. Inclusion criteria were a diagnosis of unilateral CP, age range of 12–25 years, and no history of surgery or botulinum toxin A to the upper limb in the last 12 months. Exclusion criteria were co-morbidities that would limit the participants' completion of the study and the presence of contraindications to MRI (e.g., metal implants such as teeth braces, which could distort cranial images) or TMS (history of epilepsy).

CLINICAL MEASURES

Upper limb function was assessed using the Box and Block Test and the Melbourne Assessment of Unilateral Upper limb Function (MUUL) (10, 11). Note, the MUUL has established psychometric properties for children age 4–15 years; however, it was selected as the most suitable tool to assess unimanual function for the wide age range of participants in this study. The generic functional and upper limb range of movement tasks assessed in this measure (e.g., hand to mouth or forearm supination range of motion) were considered appropriate for older age groups to complete. In addition, five bimanual everyday activities were videoed and scored by an occupational therapist blinded to other measures. Tasks were scored on spontaneous use and amount of assistance of the affected hand during the tasks, using a four-point scale with a maximum score of 30 (12). Self-reported perceptions of difficulty performing daily activities (mainly bimanual) were assessed by using the condition-specific Abilhand-Kids questionnaire (13). The "Kids" version was used for all participants. This version has only been validated in children with CP from 6 to 15 years, but the functional tasks were considered to be appropriate for participants over 15 years and the adult version is not specific to the CP population. The total score was converted to logits for statistical analysis creating an interval score on a continuum (http://www.rehab-scales.org/abilhand-kids.html; last accessed 20/03/2014). Hand function was classified using the Manual Ability Classification System (MACS) (14) and the presence of mirror movements during simple hand tasks was classified on a 0–4 scale, with 0 = no imitative movements through to 4 = movements are equal to that expected of the intended hand, as described by Woods and Teuber (15).

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging brain scans were acquired with a General Electric, HDx 3 Tesla system. Sagittal and high resolution 3-D T_1, diffusion-weighted, axial FLAIR, and T_2 weighted diagnostic imaging was completed to assess the extent of the brain lesion. Structural T_1-weighted images were acquired with 3D, sagittal sequence of 128 contiguous slices [TR = 11 ms, TE = 4.5 ms, Field of View (FOV) = 240 mm; Inversion time 450 ms and voxel dimensions of 0.47 mm × 0.47 mm × 1.3 mm). Blood oxygenation level-dependent (BOLD) contrast images were obtained using a T_2 weighted gradient echo EPI sequence of hand motor activity interspersed with rest periods to map hand motor cortical localization (TR = 4 s, TE = 35 ms, FOV = 240 mm, voxel dimensions 3.75 mm × 3.75 mm × 4 mm). Diffusion-weighted imaging was acquired with a spin echo EPI pulse sequence (TR = 8.3 s, FOV = 240 mm, TE = 87.3 ms, and voxel dimensions 0.94 mm × 0.94 mm × 5 mm) with 25 uniformly distributed Stejskal–Tanner motion-probing gradient orientations ($b = 1000$ s/mm^2) and two $b = 0$ images. All images were reported by a neuroradiologist, blinded to other aspects of the study. Lesions were classified into three categories: cortical–subcortical (C/SC); Periventricular (PV); Malformations (MAL) (16, 17).

During the fMRI experiment, participants completed a self-paced hand grip and release task, using a MR-compatible device. There were three conditions (bimanual, left only, right only) presented in a block design interspersed with rest periods (six cycles per condition; 24 s on and 24 s off). For unimanual trials, the non-active hand was strapped into a resting hand splint. A constant visual cue remained on screen to indicate the rest period. An additional visual cue flashed on every second during the hand motor activity period. Functional images were slice time corrected, realigned, smoothed, and co-registered using FSL/FEAT (http://www.fmrib.ox.ac.uk/fsl/) (18). The difference in BOLD signal between rest and movement condition (bimanual, left, right) was quantified by voxel cluster analysis, using an activation threshold of ($z = 2.3$, $p < 0.05$) bilaterally in the relevant regions of interest (ROI) of primary and premotor cortex and supplementary motor area, derived from the Juelich histological brain atlas (19). A laterality index (LI) was calculated with the equation below to quantify BOLD activation between hemispheres for each ROI during the affected hand movement as $LI = [PAV_{(CL)} - PAV_{(IL)}]/[PAV_{(CL)} + PAV_{(IL)}]$, where PAV = percentage of active voxels, and IL and CL are ipsilesional (side of the lesion) and contralesional (CL) hemispheres. LI ranges between −1.0 and +1.0, where −1.0 indicates only ipsilesional activity and +1.0 indicates only CL activity. All MR measurements were completed by one experimenter (AM) using a semi-automated pipeline in FSL/FEAT program and manual calculation of LI.

Diffusion-weighted MRI analysis was completed using the FSL/FDT processing pipeline to characterize the white matter tracts within the brain (20). This included distortion correction (eddy current correction), brain extraction, tensor fitting, and establishing scalar files, i.e., Fractional Anisotropy (FA) RGB file. DW–MR imaging data from participants no. 13 and 16 were excluded as excessive head motion during collection resulted in an inability to complete distortion correction or brain masking.

Fractional anisotropy values were determined bilaterally for a region outlining the posterior limb of the internal capsule (PLIC) (21). The PLIC was defined in MRIcro (www.mricro.com)

from the level of the anterior commissure to the base of the corona radiata (22) and using the FA RGB files (Note, intra or inter-rater reliability was not completed for determination of the PLIC). An FA asymmetry index (FAAI) was calculated as $FAAI = (FA_{CL} - FA_{IL})/(FA_{CL} + FA_{IL})$. FAAI ranges from −1.0 to +1.0, with positive values indicating reduced FA in the ipsilesional PLIC and 0 indicating symmetrical FA between PLICs (21).

TRANSCRANIAL MAGNETIC STIMULATION

Single pulse TMS of M1 was delivered with a MagStim 200 stimulator (MagStim Company, Dyfed, UK) and a figure-of-8 coil (wing diameter 9 cm) orientated to induce posterior–anterior current flow in M1. MEPs were recorded from surface EMG in bilateral first dorsal interosseus (FDI) and extensor carpi radialis (ECR). The optimal site of stimulation was determined for each target muscle, facilitated by the use of a stereotaxic camera system and Brainsight™ (Rogue Research, Montreal). Rest motor threshold (RMT) was determined in FDI using conventional procedures (23). MEPs were deemed absent if MEP amplitude was less than 50 μV with 100% muscle stimulator output (MSO). Average MEP amplitude and latency were determined from 8 responses to TMS at 120% RMT. The CL hemisphere was stimulated first prior to assessment of responses to stimulation of the ipsilesional hemisphere.

Intracortical and interhemispheric inhibition were examined from measures of cortical silent period (cSP) and ipsilateral silent period (iSP), respectively. The cSP was determined for each hand separately, during wrist extension and a pincer grip contraction at 50% maximal voluntary contraction (24) with TMS at 60% MSO applied to the contralateral "hotspot" for the contracting target muscle. The cSP duration was estimated from the stimulus onset to the return of EMG. The average cSP was calculated from 12 trials. The iSP indicates an inhibitory influence of the stimulated M1 on the opposite M1, mediated at least in part via the corpus callosum. TMS was applied to the ipsilesional hemisphere at 80% MSO while the participant maintained voluntarily hand contraction as above. The iSP was determined in FDI EMG between 30 and 80 ms post stimulus by detecting the region below one third of the average root mean square amplitude of pre-trigger EMG (25). The iSP duration and area were identified from the average of 12 rectified EMG traces, as was the persistence or number of trials that produced an iSP (26).

STATISTICAL ANALYSIS

To examine the relationship between hand function measures and the other independent variables, parametric and non-parametric tests were performed using SPSS (V.19, IBM) dependent on the normality distribution of the data. Tests included correlation analysis using Pearson r or Spearman rho; analysis of variance (ANOVA or Kruskal–Wallis) tests, with multiple comparison post tests and independent sample tests using Mann Whitney. A stepwise multiple linear regression analysis determined which independent variables (Type of lesion, LI, FAAI) were associated with clinical upper limb function (MUUL). The level of significance was set at $\alpha = 0.05$ for all analyses. Means and standard deviations are reported unless otherwise stated.

RESULTS

UPPER LIMB FUNCTION AND CLASSIFICATION

Table 1 summarizes the individual and group mean clinical assessment scores. The mean MUUL score was 80% (range 41–100%), with a strong correlation found between the MUUL and other capacity based upper limb function measures (Box Block: $r = 0.81$; Bimanual: $r = 0.83$; $p < 0.0001$). There was a weak association between MUUL and self-reported difficulty in performing upper limb daily activities determined by the Abilhands ($r = 0.42$, $p = 0.05$). Higher performances on all tasks were found in the MACS level I group [ANOVA: MUUL: $F(1, 19) = 31.05$, $p < 0.001$; Box Block: $F(1, 19) = 23.8$, $p < 0.0001$; Bimanual: $F(1, 19) = 13.7$, $p = 0.0001$; Abilhands: $F(1, 19) = 7.03$, $p = 0.02$]. Six participants had Grade 2 evidence of slight mirror movements during finger tapping, opposition, and hand grip tasks (15).

CLASSIFICATION OF LESION

Lesion characteristics are summarized in **Table 1**. All participants completed the scanning protocol for the purposes of lesion characterization. There was a difference in upper limb function scores dependent on lesion classification [ANOVA, $F(2, 17) = 42.48$, $p < 0.0001$]. Participants with C/SC lesions had lower mean MUUL (mean MUUL = 57 ± 11%) compared to those participants with PV or MAL (mean MUUL 96 ± 5% and 84 ± 9%, respectively). Participants with C/SC lesions had more impaired upper limb function than the other lesion types, as measured by Box and Block [ANOVA, $F(2, 17)$ 20.10, $p < 0.001$], Bimanual tasks [ANOVA, $F(2, 17)$ 8.60, $p < 0.001$], and Abilhand-Kids questionnaire [ANOVA, $F(2, 17)$ 8.11, $p < 0.001$].

FUNCTIONAL MRI

Performing the fMRI task with the affected hand revealed the expected BOLD activation primarily around the "hand knob" of the precentral gyrus of the ipsilesional hemisphere for 16 participants (27). However, four participants had motor area activation predominantly within the CL hemisphere. **Figure 1** shows statistical parametric maps of three participants with distinct activation profiles.

During affected hand movement, the mean LI was −0.53 (range −1.0 to 1.0) for primary and pre motor cortex (**Table 1**) and 0.00 (range −1.0 to 1.0) for supplementary motor area, with a negative LI indicating greater activity in the appropriate ipsilesional hemisphere. Participants 12, 16, 17, and 20 had activity lateralized toward the CL hemisphere (LI = 1.00). Note, these four participants also all had Grade 2 or slight mirror movements detected on clinical assessment. Participant 14 had near symmetrical activity (LI = −0.05). LI was not associated with any measure of upper limb function (MUUL: $r = -0.25$, $p = 0.20$) or lesion classification [ANOVA, $F(2, 17) = 0.26$, $p = 0.76$].

DIFFUSION-WEIGHTED MRI

DW–MRI data from two participants (no. 13 and 16) were not usable because of excessive head motion. The mean FA of the ipsilesional (affected) hemisphere PLIC was 0.45 ± 0.14 and in the unaffected hemisphere 0.56 ± 0.08. The group average FAAI was 0.15, range −0.02 to 0.69 (**Table 1**), with the positive FAAI indicating a reduced anisotropy in the ipsilesional

Table 1 | Participant characteristics.

No.	Age	Sex	Hemi	Lesion	fMRI	DTI	TMS		Upper limb function measures					
					LI	FAAI	MEP present affected hand IL stim	MEP present affected hand CL stim	MACS	Mirror	MUUL (%)	Box block	Bimanual tasks	Abilhands (logits)
1	18	F	R	PV	−0.96	0.04	Y	N	I	–	100	45	30	6.68
2	18	F	R	PV	−1.00	−0.02	n/a	n/a	I	–	95	38	30	5.04
3	16	M	R	PV	−0.26	0.07	Y	N	I	–	98	45	30	6.68
4	12	M	R	PV	−0.80	0.06	n/a	n/a	I	–	98	35	30	6.68
5	22	F	L	PV	−1.00	0.18	n/a	n/a	I	–	86	24	23	6.68
6	13	M	L	PV	−1.00	0.00	Y	N	I	–	99	46	30	5.04
7	16	M	L	PV	−1.00	0.21	n/a	n/a	I	–	92	18	16	6.68
8	16	F	R	PV	−1.00	0.01	Y	N	I	–	100	29	30	6.68
9	14	M	R	PV	−1.00	−0.02	Y	N	I	–	100	45	30	6.68
10	18	F	R	C/SC	−1.00	0.69	n/a	n/a	II	–	56	3	20	1.76
11	12	M	L	C/SC	−1.00	0.03	Y	Y	II	–	64	17	12	3.9
12	13	M	R	C/SC*	1.00	0.18	N	Y	II	Y	71	19	22	0.51
13	15	M	L	C/SC*	−1.00	*n/a*	n/a	n/a	II	–	41	4	10	6.68
14	13	M	L	C/SC	−0.05	0.42	N	Y	II	–	46	13	16	3.51
15	12	F	L	C/SC	−1.00	−0.01	n/a	n/a	II	Y	70	14	22	2.89
16	12	F	L	C/SC	1.00	*n/a*	n/a	n/a	II	Y	51	6	19	3.9
17	21	M	L	MAL	1.00	0.11	N	Y	I	Y	89	19	23	3.51
18	14	F	L	MAL	−0.54	0.11	Y	Y	II	Y	74	16	18	5.04
19	13	F	R	MAL	−1.00	0.07	Y	N	II	–	93	20	30	1.96
20	17	M	R	MAL	1.00	0.31	N	Y	II	Y	79	18	20	6.68
Mn	15	–	–	–	−0.50	0.15	–	–	–		80	24	23	4.86
SD	3	–	–	–	0.79	0.19	–	–	–		20	14	7	2.00
Min	12	–	–	–	−1.00	−0.02	–	–	–		41	3	10	0.51
Max	22	–	–	–	1.00	0.69	–	–			100	46	30	6.68

Hemiplegia (R) right; (L) left = affected upper limb.

*Lesion: PV, periventricular; C/SC, cortical/subcortical; MAL, malformation; *evidence of bilateral lesions.*

LI, laterality index derived from functional MRI.

FAAI, fractional anisotrophy asymmetry index derived from diffusion-weighted imaging.

MEP (IL), motor-evoked potentials in FDI muscle of affected upper limb, from ipsilesional (IL) transcranial magnetic stimulation.

MEP (CL), motor-evoked potentials in FDI muscle of affected upper limb, from contralesional (CL) transcranial magnetic stimulation.

(Y) Yes, present; (N) No, absent; n/a, not applicable – did not have TMS due to contraindications.

MACS, Manual Ability Classification System.

MUUL, Melbourne assessment of unilateral upper limb function (%).

Mirror = mirror movements, Y = grade 2 presence of slight mirror movement (15).

Mn, mean; SD, standard deviation; Min, minimum; Max, maximum.

PLIC compared to the CL side. There was a strong association between FAAI and upper limb function assessments (MUUL: $r = -0.67$, $p = 0.002$; Box Block: $r = -0.65$, $p = 0.003$; Bimanual tasks: $r = -0.49$, $p = 0.03$). Participants with better function had more symmetrical FA between the ipsilesional and CL PLIC. FAAI was not strongly related to the BOLD LI values ($r = 0.24$, $p = 0.30$), although, there was a trend for participants with a shift in fMRI activity to the CL hemisphere to have greater asymmetry in FAAI. There was no difference in the FAAI based on lesion classification group [ANOVA, $F(2, 15) = 2.30$, $p = 0.13$], although there was a trend for greater asymmetry in participants with cortical and subcortical lesions (mean FAAI = 0.26 for C/SC lesions; 0.15 for MAL; 0.06 for PV).

TRANSCRANIAL MAGNETIC STIMULATION

Eight participants could not have TMS due to contraindications (i.e., history of epilepsy). Of the 12 tested, all had MEPs in the unaffected FDI from TMS of the CL M1. Mean RMT was $55.3 \pm 14.2\%$ MSO with mean MEP latency in the unaffected FDI of 23.0 ± 2.0 ms (**Table 2**). Six of these participants showed atypical MEPs in FDI bilaterally in response to TMS of the CL M1 (**Figure 2**). MEP latency of affected FDI was 23.6 ± 1.9 ms.

Transcranial magnetic stimulation of the ipsilesional M1 yielded MEPs in the affected FDI of 8 participants, with a mean RMT $70.8 \pm 19.8\%$ MSO and MEP latency of 23.4 ± 2.0 ms (**Table 2**). Two participants had MEPs in the affected FDI with

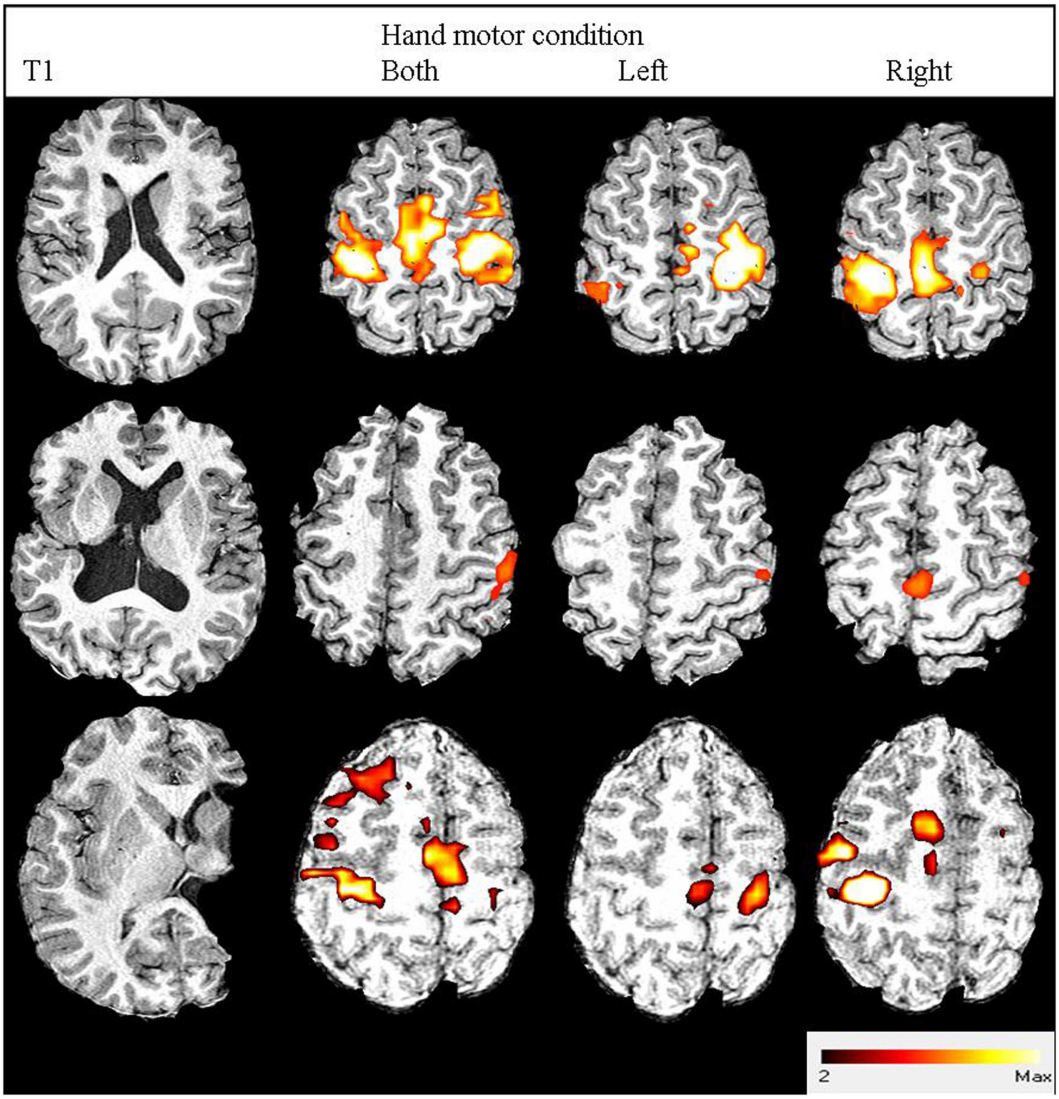

FIGURE 1 | Three participants' images from structural and functional MRI. From left to right: anatomical T1-weighted image; fMRI contrast results thresholded to $z > 2.3$: both hands active condition; left hand active condition; right hand active condition. From top to bottom: participant no. 19 with right hemiplegia and high hand function (93% MUUL) showing normal contralateral (M1/S1, PMd, PMv) and medial (SMA) activation; Participant no. 20 with right hemiplegia and average hand function (79% MUUL) showing a shift in fMRI activation lateralized to CL hemisphere for the affected right hand condition: participant no. 13 with left hemiplegia and poor hand function (41% MUUL) showing less activation than Participant no. 19 but with some remaining contralateral organization.

both ipsilesional and CL stimulation. Four participants had no MEPs in the affected FDI with ipsilesional M1 stimulation.

There was a positive trend in the relationship between the functional integrity of corticomotor pathways and measure of hand function (Kruskal–Wallis, Bimanual tasks $p = 0.01$; MUUL $p = 0.07$), with higher median MUUL and bimanual task scores for participants with normal contralateral pathways compared to those with no MEPs on ipsilesional stimulation or bilateral MEPs with CL stimulation.

All 12 participants had a cSP from CL stimulation (duration 141 ± 56.3 ms), with three participants having bilateral cSP present (duration 115 ± 63.8 ms) and correspondingly no cSP from ipsilesional stimulation (**Figure 2**). In total, only seven participants had a cSP from ipsilesional stimulation. For these participants mean cSP was 86 ± 46.8 ms. An association between the presence of CL and ipsilesional cSP and upper limb function (Mann Whitney, $p = 0.02$) was found, with higher function (median MUUL 99%) in those with cSP present from both hemisphere stimulation (**Table 2**). However, there was no association between the duration of CL cSP and upper limb function measures (MUUL, rho = 0.1).

An iSP was obtained in seven participants, with a mean duration of 22.7 ms (range 17–33 ms) and mean percentage inhibition of 15% (range −20 to 79%) (**Figure 2**). An iSP was not present in

Table 2 | Mean rest motor threshold and MEP latency (120% MSO).

Hemisphere stimulated	Rest motor threshold % (SD)	Mean MEP latency ms (SD)
Contralesional	55.3 (14.2)	Unaffected hand: 23.0 (2.0) *n* = *12* Affected hand: 23.6 (1.9) *n* = 6
Ipsilesional	70.8 (19.8)	Affected hand: 23.4 (2.0) *n* = *8*

Inhibitory function:

Hemisphere stimulated	Mean cSP duration ms (SD)	Mean iSP persistence/12 (range)	Mean iSP duration ms (SD)
Contralesional	Unaffected hand: 141 (56) *n* = *12* Affected hand: 115 (64) *n* = *3*	–	–
Ipsilesional	Affected hand: 86 (47) *n* = *7*	8 (4–11)*n* = *7*	22.7 (5.4) *n* = *7*

SD, standard deviation; MEP, motor-evoked potentials; cSP, cortical silent period; iSP, ipsilateral silent period.
Persistence = number of trials out of 12 where silent period present.

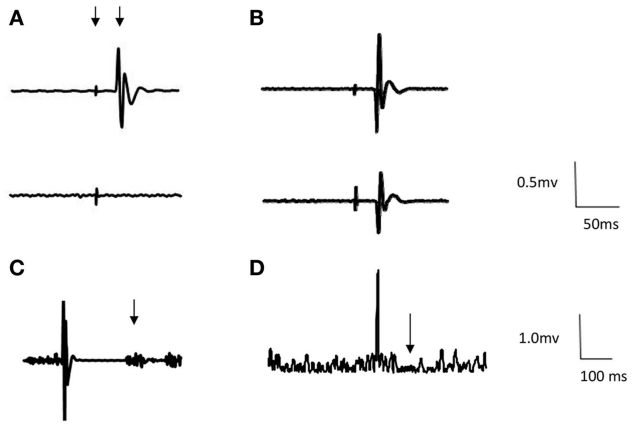

FIGURE 2 | Representative first dorsal interosseous (FDI) EMG traces from two participants. Arrows indicate stimulus artifact, MEP [example **(A)**], return of voluntary EMG [example **(C)**], and ipsilateral SP [example **(D)**]. **(A)** Example of appropriate response to TMS to the contralesional hemisphere. Top trace = unaffected FDI and bottom trace = affected FDI (Participant no. 1). **(B)** Example of bilateral response to TMS to contralesional hemisphere. Top trace = unaffected FDI and bottom trace = affected FDI (Participant no. 17). **(C)** Example of cortical silent period following ipsilesional stimulation (Participant no. 1). Traces are averages of 12 sweeps. **(D)**. Example of ipsilateral SP following stimulation ipsilesional hemisphere (Participant no. 1). Traces are rectified averages of 12 sweeps.

the four participants with no ipsilesional pathways, as well as two other participants. There was an association between the amount of iSP inhibition and clinical measures, with greater function in those with more effective inhibition (Kruskal–Wallis $p < 0.001$; post-test MUUL $p = 0.001$; Bimanual $p = 0.05$).

RELATIONSHIP WITH UPPER LIMB FUNCTION

Fractional anisotropy asymmetry index was the best indicator of clinical upper limb function, explaining 41% of the variance for MUUL ($R^2 = 0.41$, $F = 11.3$, $p = 0.004$). The addition of lesion type further increased predictive power ($R^2 = 0.57$, $F = 9.8$, $p = 0.02$).

DISCUSSION

The novel findings of this study are that in unilateral CP intracortical and interhemispheric inhibitory measures vary depending on the structural integrity of descending motor pathways. Generally, participants with intact intracortical and interhemispheric inhibition had higher upper limb function. Understanding the association between upper limb function, motor cortex function, and motor pathway integrity may assist in the targeting and development of novel upper limb rehabilitation strategies in youth with unilateral CP (4).

This is one of the first studies of unilateral CP to describe M1 inhibitory function by evaluating the cortical (cSP) and ipsilateral (iSP) silent periods. Silent period measures reflect activity of $GABA_B$-ergic cortical inhibitory mechanisms (24). In neurologically unimpaired individuals, cSP duration can be greater than 200 ms (24). In this study, the duration of the cSP was less than these previously reported values in both the CL (141 ± 56 ms) and ipsilesional hemispheres (86 ± 42 ms), with only 7 out of 12 participants showing a cSP with ipsilesional stimulation. These results are similar to a previous study in a CP sample that found reduced cSP durations in the lower limb in children with spastic diplegia (28).

The iSP is mediated, at least in part, by transcallosal pathways and reflects the ability of the ipsilesional hemisphere to inhibit the CL hemisphere through GABA-ergic interneurons (24). The iSP in a neurologically intact population has been shown to have an average duration of between 25 and 33 ms (26, 29). In the current study, the average iSP duration was 23 ms, but there was wide variation in the amount of inhibition and 5 of the 12 participants assessed did not show any iSP. In the adult stroke population, a reduced interhemispheric inhibition from ipsilesional to CL primary motor cortex is common, affecting the balance of excitability between the hemispheres (30). Furthermore, interventions that restore balance in hemispheric excitability by modulating intracortical and interhemispheric inhibition have been associated with better outcomes (30). Enhancing interhemispheric inhibition is a potential mechanism that could be targeted in unilateral CP. It remains to be determined if this would improve outcomes for these participants.

The three main types of brain lesions in the current sample (PV; MAL; cortical/subcortical), represent the most frequent structural lesions described in unilateral CP (16). In our study, upper limb function was related to lesion type, as observed previously, where individuals with PV lesions had better hand function than individuals with cortical, subcortical, or acquired lesions (16, 31). Staudt and colleagues also found that individuals with larger PV lesions and those with lesions developing late within the third trimester (predominantly cortical and deep gray matter), had worse hand function (8). In contrast, others have found no correlation between lesion type and motor impairment (32). This difference might be partially explained by the use of non-standardized measures of hand function in previous studies. Lesion volume has also been previously shown to impact function (5), though this was not explored in this current study, in which the focus was on corticospinal tract integrity specifically.

Among the five participants who showed motor pathway reorganization, there was a trend toward more impaired upper limb function, similar to previous findings (8). fMRI data indicated these participants had a shift in the cortical control of the affected hand toward the CL hemisphere. These individuals had either a cortical/subcortical lesion ($n = 3$) or malformation ($n = 2$) and a spread of upper limb function mostly across the lower range. Holmstrom et al. (33) also found the lowest levels of hand function in individuals with reorganization to the CL hemisphere ($n = 5$) (33). However, there was again variation in ability across these individuals indicating that there may be potential for some individuals with CL reorganization to attain a good level of upper limb function. In general, the small number of participants with CL reorganization is a limitation in both our work and previous studies and remains a topic of further enquiry (7, 9).

Hemisphere laterality of motor activity, as determined from fMRI, was not related to hand function measures for the participants in this study, potentially reflecting the variability in the group described above. Functional MRI is a useful tool in detecting corticomotor re-organization and our results confirm earlier studies that contralateral control is not a default mode of upper limb function in this population (7, 34). However, TMS and motor-evoked potentials may offer a more in depth assessment of corticomotor function, specifically within M1.

Interestingly, descending white matter pathway integrity, as determined by asymmetry of FA measurements in the PLIC was strongly related to the upper limb functional measures. This is consistent with Holmstrom et al. (35) who also found mean FA values in the PLIC of children with unilateral CP correlated to the Box and Block measure of gross motor hand function (35). In children with CP, PLIC FA may be able to provide information that anatomical MRI alone cannot provide, such as a quantitative threshold level to indicate whether sufficient motor pathways are intact for particular interventions (36). As such, adopting DW–MRI provides knowledge of the specific pathophysiology and may be useful to guide treatment planning away from a "one-size fits all" treatment approach (37).

As a progression from this, recent work has found variable outcomes in the association of MRI findings and efficacy of CIMT in children with unilateral CP. Three recent small studies found no relationship between the amount of improvement following a CIMT program and brain lesion characteristic or organization (38–40), with three other studies finding imaging information did have a predictive ability for the efficacy of a CIMT program (4, 41, 42). However, authors caution at this early stage that generalization of results is not possible due to the small sample sizes (39).

LIMITATIONS

Unilateral CP is characterized by a high degree of heterogeneity in terms of lesion type making it difficult to generalize findings from individual studies; the future use of meta-analyses may allow stronger conclusions. In particular, the TMS results in this study were taken from a smaller sample of just 12 participants. This study was unique in that it used a wide range of standardized upper limb functional measures and classifications to relate to neuroimaging and neurophysiological measures. This supports the recommendation from a recent systematic review on use of DW–MRI in CP on the inclusion of standardized functional measures, such as the Melbourne UUL, to allow for future meta-analysis (43).

Both fMRI and some TMS techniques are more appropriate in the older child, due to active participation and tolerance of the procedure. However, quantitative assessment using DW–MRI has potential to be added to routine structural MRI in younger children. However, the ability to complete standardized analysis of this information to fit into a clinical setting remains a challenge (43). No attempt was made to determine intra or inter-rater reliability of the imaging-based measures, which is a potential limitation.

CONCLUSION

These results indicate that DW–MRI and TMS measures provide a useful addition to standard MRI and relate to upper limb function. Such techniques may help in individualizing therapy, based on characteristics such as motor pathway integrity and reorganization profile. This paper serves to improve our understanding on the inter and intra hemispheric inhibitory mechanisms operating in children with unilateral CP, with potential for these mechanisms to become a target of intervention, similar to what is now being explored in the adult stroke literature.

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Stem cell therapy for neonatal hypoxic-ischemic encephalopathy

Gabriel S. Gonzales-Portillo†, Stephanny Reyes†, Daniela Aguirre†, Mibel M. Pabon and Cesar V. Borlongan*

Department of Neurosurgery and Brain Repair, University of South Florida, Tampa, FL, USA

Edited by:
Anna Purna Basu, Newcastle University, UK

Reviewed by:
Seongjin Yu, NHRI, Taiwan
Takao Yasuhara, Okayama University, Japan

***Correspondence:**
Cesar V. Borlongan, Center of Excellence for Aging and Brain Repair, University of South Florida College of Medicine, 12901 Bruce B Downs Blvd, Tampa, FL 33612, USA
e-mail: cborlong@health.usf.edu

†Gabriel S. Gonzales-Portillo, Stephanny Reyes and Daniela Aguirre have contributed equally to this work.

Treatments for neonatal hypoxic-ischemic encephalopathy (HIE) have been limited. The aim of this paper is to offer translational research guidance on stem cell therapy for neonatal HIE by examining clinically relevant animal models, practical stem cell sources, safety and efficacy of endpoint assays, as well as a general understanding of modes of action of this cellular therapy. In order to do so, we discuss the clinical manifestations of HIE, highlighting its overlapping pathologies with stroke and providing insights on the potential of cell therapy currently investigated in stroke, for HIE. To this end, we draw guidance from recommendations outlined in stem cell therapeutics as an emerging paradigm for stroke or STEPS, which have been recently modified to Baby STEPS to cater for the "neonatal" symptoms of HIE. These guidelines recognized that neonatal HIE exhibit distinct disease symptoms from adult stroke in need of an innovative translational approach that facilitates the entry of cell therapy in the clinic. Finally, new information about recent clinical trials and insights into combination therapy are provided with the vision that stem cell therapy may benefit from available treatments, such as hypothermia, already being tested in children diagnosed with HIE.

Keywords: cerebral palsy, stem cells, hypothermia, combination therapy, translational research

CLINICAL MANIFESTATIONS OF NEONATAL HYPOXIC-ISCHEMIC BRAIN INJURY

Hypoxic-ischemic encephalopathy (HIE), cerebral palsy (CP), and periventricular leukomalacia (PVL) are mainly triggered by neonatal hypoxic-ischemic brain injury. Neurodevelopmental deficits such as learning disabilities, mental retardation, and hearing and visual impairments accompany children diagnosed with hypoxic-ischemic brain injury. Brain expression of systemic asphyxia characterizes HIE (1). Perinatal asphyxia and resulting hypoxic-ischemic encephalopathy (HIE) occur in 1–3 per 1000 births in the United States (2). Worldwide, 10–60% of infants who develop HIE will die and at least 25% of the survivors will have long-term neurodevelopmental sequelae (2). Hypoxic-ischemic encephalopathy is the primary cause of 15–28% of cerebral palsy among children (2). Throughout the paper, the terms HIE and the alternative term neonatal encephalopathy (NE) (3, 4) are synonymous. These two terminologies have been a topic of much debate (5, 6). Even with an intense effort by researchers and clinicians to employ precise diagnostic methods, encephalopathy has not been identified in premature infants as compared to full term infants (7–9). HIE brings a relatively high 50% mortality rate in newborns (10), and a small portion of those survivors, 25% display CP symptoms permanently (11, 12). Ischemic perinatal stroke is responsible for 30% of children with CP (13). A cerebral white matter injury, known as PVL, is detected in 50% of neonates with exceedingly low birth weights with 90% of survivors displaying CP symptoms (14); however, studies using ultrasonography report findings of the incidence of PVL to be lower than 50% (15–17). As a result of the very similar pathophysiological symptoms between neonatal hypoxic-ischemic brain injury and adult stroke, innovative treatments such as cell-based therapies, which are currently being tested in stroke, may prove to be successful in neonatal hypoxic-ischemic brain injury. Having a grasp of the neurochemical cascade of events is a holy grail for commencing treatment intervention in neonates (18). To this end, therapeutic benefits may be achieved by abrogating the "secondary energy failure" or "excite-oxidative cascade" (18, 19). This is characterized by amplified excitation of NMDA receptors combined with peculiar oxidative stress due to mitochondrial dysfunction, altogether depleting energy from the brain seen in infants with hypoxic-ischemic injury (18). Currently, hypothermia is used to treat HIE (20–22) and has demonstrated to be very effective in newborns with a gestational age of $\geq$36 weeks (22, 23) diagnosed with moderate to severe HIE (21, 22), but neurodevelopmental deficits persist in 40–50% of patients even after hypothermia (22). A treatment that combines both hypothermia and cell transplantation may prove to be more effective and benefit neonates with moderate to severe HIE (**Figure 1**).

Abbreviations: CP, cerebral palsy; HI, hypoxic-ischemia; HIE, hypoxic-ischemic encephalopathy; MSCs, mesenchymal stem cells; NE, neonatal encephalopathy; PVL, periventricular leukomalacia; STEPS, stem cell therapeutics as an emerging paradigm for stroke.

KEY PRECLINICAL GATING ITEMS FOR STEM CELL THERAPY FOR HIE

Academicians, industry partners, and regulators, which include both the National Institutes of Health (NIH) and the U.S. Food and Drug Administration (FDA), have jointly created Stem cell Therapeutics as an Emerging Paradigm for Stroke (STEPS). Together, they have provided guidelines to increase the successful outcome

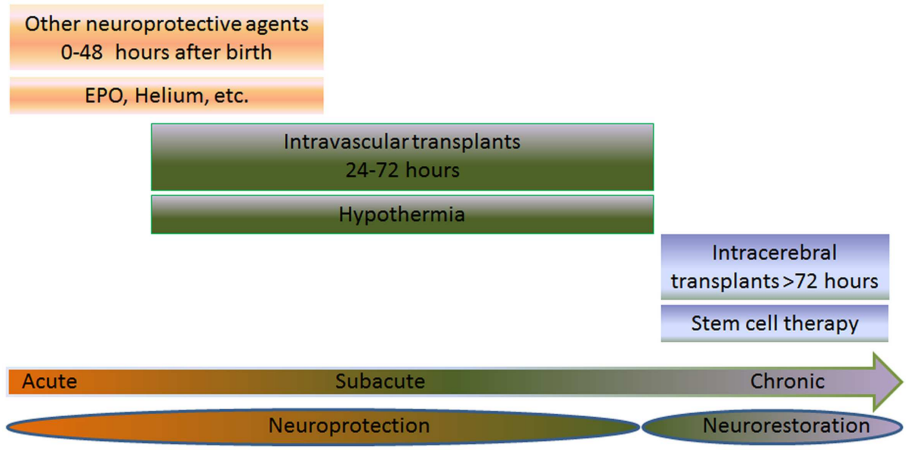

FIGURE 1 | Multiple intended combination therapies are shown for three different groups: acute (0–48 h after birth), subacute (6–72 h after birth), or chronic (>72 h after birth). The joint effort of both cell transplantation therapy and neuroprotective therapies, such as hypothermia, erythropoietin (EPO), and helium are used for treating neonates with HIE. When treating the neonate in the acute or subacute phase, the treatment is referred to as neuroprotection. On the other hand, treatments aimed at the chronic stage are referred to as neurorestoration. EPO or helium is used to treat the subject during the acute phase, while hypothermia and stem cell therapy is used to treat the subacute stage via intravascular routes. When treating the chronic stage, stem cell therapy is used via intracerebral route, which stimulates the neurorestorative mechanisms. The combination of these therapies may prove to be effective in neonates suffering from HIE.

of cell therapy in stroke patients (24–31). The need for the establishment of a Baby STEPS consortium is necessary for younger patients (32). This would allow a safe and effective translation of cell therapy in neonatal hypoxic-ischemic brain injury. Below, we identify critical gating criteria in conducting translational studies in order to aid and advance the formation of Baby STEPS guidelines.

CLINICALLY RELEVANT MODELS OF HIE

The animal species and strain should be specially considered in HIE modeling. Rodent models such as Vannucci's model of neonatal hypoxic-ischemic brain injury parallel many pathological events that humans endure during neonate HIE (33). Researchers found that in 7-day-old postnatal rats, which undertook ligation of unilateral carotid artery as well as systemic hypoxia, suffered widespread cell death to cerebral cortex, subcortical and periventricular white matter, striatum, and hippocampus ipsilateral to the ligated artery (34). Rats are not the only species used to create the Vannucci model, but mice have also been used (35) with varying pathological outcomes dependent on the mouse strain (36–39).

Another equally important variable that should be controlled in the experimental HIE is the age of the animals. Younger animals have shown to be impervious against hypoxia. A 1–2-day-old postnatal rat needs to be exposed to more severe hypoxia as compared to its 7-day-old counterpart in order to attain efficacious HIE symptoms (40). Another important fact to note is that younger animals experience worse white matter injury than older rats (35). Therefore in HIE modeling, age is a critical factor and is further verified by a focal subcortical cell loss paired with a surge in proliferating oligodendrocyte progenitor cells following HIE in young neonates, but rather modest in older models (41–45). Age-related changes following HIE need to become standardized in order to better evaluate the therapeutic benefits of experimental treatments.

Gender should also be taken into consideration for models, in that HIE-induced female neonates displayed a much smaller infarct volume and improved sensorimotor task than their male counterparts after perinatal hypoxic-ischemic brain injury and treatment with erythropoietin (46). A possible explanation behind the differences between female and male neonatal infarct sizes and improved neurological behavior has become increasingly clear. It is known that gender differences in injury to the brain are not merely a result of hormonal influence (47), but the properties of individual cells (48). For instance, male and female cells display differential gene expression even when no hormonal influences are apparent (49), and brain cells show phenotypic differences that are gender dependent but independent of gonadal phenotype (50). Moreover, gender modulates responsiveness to recombinant erythroprotein (Epo) (51). Additionally, Epo receptor (EpoR) alleles, EpoRA1, and EpoRA10, have displayed a significantly higher frequency in females when compared to males (52). Epo administration is known to produce significant long-term neuroprotective benefit on the developing brain (46). This suggests that Epo has a gender preference with neonatal benefit in females, whose mechanisms must be further investigated. Together with other studies demonstrating that gender similarly affects injury models (53, 54), these studies suggest that gender should be carefully considered in experimental HIE.

The approximation of the clinical pathology of HIE is a crucial goal in standardizing the animal models because a model that better mimics a human condition would allow for better evaluations of possible treatments in human patients. Rats along with many other species, which include non-human primate, sheep, lamb, puppy, piglet, and rabbit, have been employed as models to closely

resemble some HIE pathological aspects in humans (33, 55–60). Unfortunately, because of the expense in using large animal models, it has deterred research with these clinically relevant models. The piglet model is a good model for research regarding treatment plans for neonates, as it closely resembles the weight and size of a newborn infant. This piglet model also reveals new treatment variables, in that phosphorylated metabolites are temperature-sensitive and that the more severe the energy depletion the worse the secondary energy failure, exacerbating neuronal death (61, 62). These findings suggest the need to control temperature and maintain brain injury in experimental models using therapeutic strategies.

CLINICALLY RELEVANT EXPERIMENTAL PARADIGMS

Lab-to-clinic translatable functional tests need to be developed to better assess the pathological improvements of experimental interventions for short and long-term outcomes that are species specific and mimic the human condition. Despite efforts of investigators to control many variables in experimental HIE models, characterizing the phenotype of encephalopathy in neonates has proven to be elusive. As opposed to adults, neonatal encephalopathy is prevented from implementation of timely interventions as there is a scarcity in studies determining the optimal supra-acute to chronic therapeutic window in laboratory models, thus presenting a major barrier to translating experimental treatments to clinical applications. Acknowledging this research gap is pivotal when designing therapeutic intervention studies for future clinical applications in neonates. In order to create well-designed translational studies, it is critical to optimize the dosage, delivery route, and timing of stem cell transplantation within applicable clinical parameters. Treating the laboratory as the clinical setting for cell therapy in HIE will enhance the translational potential of the stem cell product. In order to prevent any potential microembolism, the minimum therapeutic cell dosage must be determined. Finding minimally invasive procedures for cell delivery could prevent exacerbation of the already injured brain. For timing of cell delivery, consideration should be given to the neuroprotective phase (< 1 day of injury) and the neurorestorative phase (> 1 day after injury) (63, 64). While most transplant studies have examined a single bolus injection of stem cells, we recognize that HIE is a disorder that involves progressive neuronal cell loss even days after HI. As a result, multiple treatments of stem cells and adjunct neuroprotective therapies are more likely to have not only neuroprotective but also neurorestorative effects. This would provide a continual targeted regime to prevent cell death in the hopes to improve neurodevelopmental outcome. For example, two MSC injections at 3 and 10 days after neonatal hypoxia-ischemia (HI) markedly improved sensorimotor function 4 weeks after the insult (65). This MSC-3 + 10 treatment was more powerful in improving functional outcome and in reducing gray and white matter loss than a single MSC injection at 3 days after HI (65).

WELL-DEFINED PHENOTYPIC MARKERS OF DONOR STEM CELLS

A detailed description of the distinguishable phenotypic features of stem cells is an integral component of understanding the biology and potential of stem cells (66–74). To determine the safety and effectiveness of stem cells that are to be transplanted in HIE, it is important to know their identity. This will also give way to understanding the mechanisms allowing the functional amelioration that takes place after transplantation. That different cell types in the brain undergo degeneration has prompted the notion that brain repair consists of several mechanisms facilitating various types of cells working together with the trophic, neurogenic, vasculogenic, and other by-stander effects of the transplanted stem cells (75). Accordingly, such multi-pronged cellular repair process necessitates the need to determine the source of the stem cells to aid in realizing the therapeutic effects and mechanisms of action associated with cell therapy. Another aspect to consider is the ability of the cells to be shipped frozen and thawed for transplantation at the clinic, which is important for neonatal diseases which may benefit from early intervention. Moreover, using autologous stem cells can be of an advantage due to their ability to bypass graft rejection along with its side effects. For example, in a study where intravenous injection of autologous cord blood was made, the results showed that they were safe in CP children (76). In parallel, placental tissue obtained during prenatal chorionic villous sampling or at delivery can be a good source of autologous stem cells which can be transplanted during the last month of gestation or the first few months after delivery if neurodegeneration is detected in the baby (77).

FUNCTIONAL OUTCOMES IN CELL TRANSPLANTATION IN HIE

To assess the safety and efficacy of the treatment group in HIE, specific behavioral and histological procedures are frequently used. These assays can determine motor and cognitive amelioration as well as provide insight to the biochemical processes that are undergoing in the brain (68, 78, 79). However, to value the functional improvement after cell transplantation in animals, a close estimate of the HIE clinical symptoms needs to be shown in these preclinical models (80–83). In addition, monitoring the safety and efficacy of stem cells over time will allow a better assessment of the effects of cell therapy (84). This becomes an obstacle for neonatal HIE due to the spontaneous amelioration seen in the development and maturation phases of the neonatal animal (85) and in pediatric patients (86). More sensitive functional outcomes may be needed to delineate spontaneous recovery from true therapeutic benefits of cell therapy; for example, to examine the grafted cells and host HIE microscopically, specific markers for the trophic factor effect, immunomodulatory response, neurogenesis, vasculogenesis, angiogenesis, and synaptogenesis, as well as inflammation, tumorigenesis ,or ectopic tissue formation may be used (87–89). This approach allows a better understanding of the cells' mechanism of action and indication of unfavorable side effects.

MECHANISMS OF ACTION UNDERLYING CELL THERAPY

Two major modes of action are involved in stem cell-mediated functional recovery in ischemic brain injury: cell replacement and by-stander effect. Cellular and molecular neurorestorative mechanisms include neurogenesis, angiogenesis, synaptogenesis, immunomodulation, and trophic factor secretion (30, 31, 90). Real-time visualization techniques (i.e., magnetic resonance imaging), originally performed in stroke and extended to HIE models (91–93), have allowed, recently, the tracking of the transplant,

as well as the imaging of the host neurorestorative mechanisms (94–100). The neurorestorative mechanism is characterized by transplantation of stem cells that serve as biobridge for the initiation of endogenous repair mechanisms (101). The transplanted stem cells pave the way between the neurogenic niche and injured brain site in order to traffic the migration of host neurogenic cells (101).

OUTSTANDING PRECLINICAL ISSUES NECESSARY FOR CLINICAL TRIALS OF CELL THERAPY IN HIE

Preclinical trials on stem cells in cerebral palsy have been conducted and have shown significant improvement in animal models. However, results have been difficult to replicate in humans since most of the studies have been on acute hypoxic injury models, which are less similar to human cases compared to chronic models. So far, clinical trials on children with cerebral palsy (CP) have suggested that neural progenitor cells (NPC), umbilical cord mononuclear cells, and mesenchymal stem cells (MSC) transplants are safe, and could improve motor and cognitive functions (102). Currently, there are three ongoing registered clinical trials on stem cells and CP on children, and two that have recently been completed.

Two of these clinical trials are currently being conducted in the United States, with the aim of testing the safety and efficacy of autologous cord blood cells. The primary outcomes should be expected by the year 2015. Although patient recruitment is not open, China intends to test the efficacy of umbilical cord MSC versus rehabilitation treatments on children with cerebral palsy. In Iran, two clinical trials have already been completed. These clinical trials studied the efficacy of multiple intrathecal bone marrow-derived CD133 cell transplantation in children with cerebral palsy. Results are not published yet (103).

It is imperative to set the parameters defining safety and efficacy of stem cell therapy in neonatal HIE clinical trials. In the United States, the Medical College of Georgia and Duke University are assessing the safety and efficacy of umbilical cord blood transplants in CP pediatric patients. The long-term efficacy of intravenous transplantation of autologous cord blood in CP children remains to be determined (76, 104). So far, autologous bone marrow-derived MSCs have also been found to be safe when transplanted, but it was only on one CP patient (105). Autologous stem cell sources have been preferred largely due to their safety profile, but cell types that have already committed to a particular lineage or niche in the brain also have the potential to be used as donor cells. In order to implement cell therapy in neonatal ischemic-injury patients, there should be sufficient evidence of safety, efficacy, and mechanism of action of the stem cells in animal models. Moreover, it has been difficult to obtain a projection of the neurologic outcomes of cell therapy in neonatal hypoxic-ischemic injury. The current reports on clinical improvement after cell therapy in children with CP or HIE should not interfere with the need for sufficient preclinical studies for the advancement of clinical trials. The guidelines mentioned in the previous sections on the Baby STEPS may also be implemented to other potential therapies for neonatal hypoxic-ischemic injury (106–109) and should be used along with the current pediatric stroke recommendations in research and treatment interventions (110–113). In the end, while autologous stem cells have shown to be safe and effective as a possible treatment for HIE, more preclinical studies, paralleled by carefully designed limited clinical trials should be conducted before moving into larger studies.

CELL THERAPY AND ADJUNCTIVE TREATMENT WITH HYPOTHERMIA

The current therapies for HIE attempt to interrupt the pathways activated by HIE. In neonates with HIE, the results have not been promising in regards to preventing the neuronal loss (114, 115). In research models of HIE, it has been shown that hypothermia reduces the release of glutamate (116), reduces the secondary energy failure (21, 61, 116–118), normalizes protein synthesis (119), and reduces the injury by free radicals (115). Small trials of hypothermia conducted on human neonates (117, 118) showed promising results, while three large trials showed improvement in the neurodevelopment in neonates with mild to moderate HIE, but showed no improvement in neonates with severe HIE (20–22). There is evidence that hypothermia may exert neuroprotection by reducing the neurodevelopmental disability at 18 months of age in newborns with either moderate or severe HIE (120). Neuroprotective approaches could reduce the initial tissue damage, however, cell therapy will still be required to repair the already damaged regions of the brain. This approach can then be implemented on neonates with moderate to severe HIE.

In normal physiological conditions, the endoplasmic reticulum (ER) and mitochondria sequester calcium when intracellular levels increase (121). Calcium enters the cell through voltage-sensitive calcium channels and agonist-operated calcium channels, which are activated by glutamate, *N*-methyl-D-aspartate (NMDA) and kainate, and quisqualate (K/Q) (121). In the ischemic cascade, the increase in hydrogen displaces calcium from intracellular proteins and intracellular calcium levels increase eventually leading to mitochondrial dysfunction (121). In addition, calcium activates intracellular proteases and depolarization occurs in the cell membrane, releasing a large number of excitatory neurotransmitters such as glutamate (121). This activates NMDA receptors persistently, causing intracellular hyperosmolarity in the postsynaptic cell eventually leading to neuronal death (121). Moreover, the ongoing sodium influx inhibits the normal magnesium blockade on NMDA receptors (121). Hypothermia significantly reduces extracellular levels of excitatory neurotransmitters (121). The release of these neurotransmitters is temperature dependent and even mild levels of hypothermia exert an inhibitory effect (121). Hypothermia promotes the survival of neurons through an interaction on glycine since NMDA receptors require the presence of glycine to be activated (121). Hypothermia significantly decreases brain glycine levels after ischemia, thus decreasing hyperexcitability by glutamate (121).

Hypothermia exerts neuroprotection in HIE against aberrant stages of region-specific brain maturation (122), blood–brain barrier (BBB) impairment (123), and apoptosis due to mitochondrial dysfunction (124). Because hypothermia is most efficacious within the first 6 h of life for the infant with moderate to severe HIE (125–127), the patient may benefit even more with the use of a combinatorial therapies (128, 129). For example, using erythropoietin and helium, both which are in clinical trials (130–132),

should be considered for in combination therapy. The treatment considerations for hypothermia in addition to combining other therapies are based on the evolving pathophysiology of neonatal brain injury, discussed by Ferriero et al. (133, 134). Using a combination of therapies may be more beneficial to tackle the activated cell death pathways; moreover, detecting it early in at-risk newborns may help prevent or reduce the disabilities following neonatal brain injury (133, 134).

As discussed above, accumulating experimental data have indicated the mobilization of bone marrow-derived stem cells, such as MSCs, in brain plasticity and therapy of HIE to the affected area (135). In the clinic, MSCs can be obtained from umbilical cord blood, adipose tissue, amniotic fluid/tissue, or menstrual blood (136). As alluded earlier, autologous MSCs may be the preferred stem cells to avoid adverse effects associated with graft rejection, but allogeneic MSCs may also be equally safe and effective because of their immature immune system, as well as their capacity to secrete anti-inflammatory factor (136). MSCs are capable of differentiation into variety of phenotype cells (130, 137) and have been demonstrated to exert a therapeutic benefit against brain injury (125). However, little is known regarding MSC treatment for HIE, especially in combination with hypothermia.

The observation that seizure onset beyond the first 12 h of life is not only common in newborns with HIE (138), but also is associated with severe brain injury (56), advances the notion of a critical relationship between the onset of neonatal seizure and initiation of the therapy. Accordingly, any treatment regimen, including hypothermia, is likely to exert benefit if initiated within 6 h after hypoxic-ischemic injury and continuing over the next 12 h or even beyond (i.e., for 72 h) (138). The mechanism underlying hypothermia remains elusive, but may include its capacity to reduce oxidative stress, energy deficit, and inflammation (139). Because of the dismal prognosis of infants with HIE, clinical enthusiasm for a novel treatment is understandable (140). Based on preclinical studies, accumulating evidence suggests that in order to treat more effectively neonatal HIE, and many other neurodegenerative diseases, combination therapy can be of great help. As ischemic brain injuries have two separate cascades of events, one immediately after injury and one persisting even weeks after, it is important to use combination therapy which can tackle both events at different times. We highlight here that hypothermia could be a great neuroprotective method implemented early in HIE, while stem cells could have a better neurorestorative approach, especially during the chronic stage of the disease, which starts days after (i.e., 72 h after birth).

As mentioned previously, combination therapy may be the best approach to treat neonatal HIE, especially when a definite therapeutic time frame has not been fully established. The use of other possible neuroprotective strategies has been studied and is believed to enhance the therapeutic effects of hypothermia by targeting different therapeutic windows and stages of HIE. Oxygen free radicals are usually elevated after hypoxic-ischemic injury. The use of antioxidants like superoxide dismutase, combined with polyethylene glycol to facilitate infiltration across the blood–brain barrier, can degrade reactive oxygen species to ameliorate the negative effects of hypoxia. Nonetheless, neuroprotection in newborn animals has only been seen when administered prior to injury. Xanthine oxidase inhibitors, like allopurinol and oxypurinol, have also shown to reduce concentrations of free radicals in infants when administered early during the recovery phase, while administration of lazeroids appears to inhibit iron-dependent lipid peroxidation in immature models of hypoxic-ischemic brain damage (141, 142). Epo appears to have a longer therapeutic window (24–48 h after delivery) compared to hypothermia. Administration of Epo leads to a reduction in white matter injury, free radical production, and glutamate cytotoxicity in neonates after a hypoxic event by increasing the system XC-expression (130, 142, 143). System XC exchanges 1 mol of cystine for 1 mol of glutamate leading to increased cellular viability (143). The therapeutic effects of hypothermia and IV melatonin in perinatal asphyxia piglets model have been reported (144). Glutamate antagonists such as MK-801 have been show to reduce brain damage after hypoxic-ischemia in neonatal animal models (141, 144). The accumulation of calcium in the cytosol is also characteristic of hypoxic-ischemia. Flunarizine, a Ca + channel blocker, has shown to have a neuroprotective effects when administered prior to injury in fetus and newborn animals (141). Magnesium sulfate has also shown to be a potential treatment to reduce newborn brain injury (in rats) by blocking the neuronal influx of Ca + within the ion channel, while it could also reduce the incidence of moderate to severe CP (141, 144). However, the use of Ca + blockers has been linked to adverse cardiovascular effects on infants, contraindicating their use. It has been observed that neuronal loss can be reduced through the administration of NOS inhibitors in immature rats; however, further studies need to be conducted in order to prove its effectiveness in other animal models (141). Neuroprotective effects after the administration of noble gases like xenon, argon, and helium 2 h post hypoxic injury in 7-day-old SD rat has been reported. Although helium seems to be neuroprotective only in mild hypoxic-ischemic injury, argon and xenon showed neuroprotection in severe hypoxic animal models by increasing Bcl-2 and Bcl-xL, as well as reduction of infarct size and long-term neurocognition (144, 145). There are still many potential neuroprotective strategies to be studied including protection by neural stem cells and the ependymal lining of the ventricles, among others, which will similarly require further investigations for HIE applications.

The use of delta opioid agonists may resemble certain physiological correlation of hibernation, including hypothermia (71), which may involve direct opioid receptor activation, as well as non-opioid mechanisms (71, 146, 147). Interestingly, delta opioids may regulate neural stem and progenitor cell proliferation and differentiation (148), and may even enhance cell-based therapeutics in *in vitro* and *in vivo* disease models (149). Our recent study (150) revealed that moderate hypothermia is efficacious in an *in vitro* model of hypoxic-ischemic injury, which was enhanced by MSC treatment. We also showed that the delta opioid system, along with other non-opioid neuroprotective processes, primarily contributes to the observed neuroprotection in HIE. Stem cell therapy using MSCs significantly improved the therapeutic outcome of moderate hypothermia. Primary rat neurons were exposed to oxygen-glucose deprivation (OGD) condition, a model of hypoxic-ischemic injury, then incubated at 25°C (severe hypothermia), 34°C (moderate hypothermia), and 37°C (normothermia) with or without subsequent co-culture with MSCs. Combination

treatment of moderate hypothermia and MSCs proved to be the optimal condition for preserving cell survival and mitochondrial activity after OGD exposure. Pharmacologic induction of hypothermia in human embryonic kidney cells (HEK293) via treatment with delta opioid peptide (DADLE) resembled moderate hypothermia's attenuation of OGD-mediated cell alterations, which were much more pronounced in HEK293 cells overexpressing the delta opioid receptor. Further, the addition of DADLE to 34°C hypothermia and stem cell treatment in primary rat neurons showed synergistic neuroprotective effects against OGD which were significantly more robust than the dual combination of moderate hypothermia and MSCs, and were significantly reduced, but not completely abolished, by the opioid receptor antagonist naltrexone altogether implicating a ligand–receptor mechanism of neuroprotection. Investigations into other therapeutic signaling pathways revealed growth factor upregulation (i.e., GDNF) and anti-apoptotic function accompanying the observed therapeutic benefits. These results support combination therapy of hypothermia and stem cells for hypoxic-ischemic injury, which may have direct impact on current clinical trials using stand-alone hypothermia or stem cells for treating neonatal hypoxic-ischemic brain injury.

The use of hypothermia as a treatment strategy is not limited to neonates. In adults, hypothermia has been regarded as a therapeutic strategy to improve the patient's survival and reduce neurologic injury after cardiac arrest (151). Small clinical trials provide evidence of hypothermia as a potential treatment for traumatic brain injury (TBI) as it significantly reduced rates of death, vegetative state, and long-term disability (152). Although not regarded as a treatment strategy for spinal cord injury (SCI), the control of pressure around the injured spinal cord along with the improved behavioral outcome in animal studies demonstrates the potential of systemic hypothermia as a method of treating acute SCI (153). When used in combination with recombinant tissue plasminogen activator, animal data also show a reduction in brain hemorrhage and blood–brain barrier disruption, indicating the synergistic potential of hypothermia in stroke (154).

In addition, the use of stem cells as a therapeutic strategy in adults has obtained much attention due to the substantial beneficial data gathered in animal and clinical studies. For example, specific disease-relevant neurons can be obtained from induced pluripotent stem cells including dopaminergic neurons for Parkinson's disease (PD), hippocampal and cholinergic neurons for Alzheimer's disease, motor neurons for amyotrophic lateral sclerosis, and inhibitory interneurons for schizophrenia (155). Embryonic stem cells can be made to also differentiate into dopaminergic neurons and have been shown to ameliorate the behavioral deficit in animal models of PD (156). Embryonic neural stem cells are also considered in demyelinating diseases, such as multiple sclerosis, to replace glial cells that have been lost (156). In addition, the use of neural stem cells in Huntington's disease has been evident (156).

SYNOPSIS

Stem cell therapy has the potential to become a treatment method for neonatal hypoxic-ischemic brain injury, but it needs to be first implemented in the clinic on patients with neonatal hypoxic-ischemic brain injury. This will require further translational research studies so that stem cell therapy can be fully implemented. Stem cell therapy can also benefit from the ongoing trials of stem cell therapy in adult stroke due to the similarities in pathology. Neonatal hypoxic-ischemic injury, however, has distinct symptoms from adult stroke (157–159) that will demand modifications to the translational approach before it can be applied to the clinic. Combining both hypothermia and stem cell therapy may further improve the results of cell therapy in neonatal hypoxic-ischemic injury. The results from using this combinatorial approach can be then measured by behavioral and histological assays. In addition, using biomarkers to monitor the transplanted cells in the patient over time will contribute to exposing the long-term effects of this combinatorial therapy.

Currently, the use of stem cells for neonatal hypoxic-ischemic brain injury is still in its experimental phase. Although clinical trials are scheduled to be conducted using autologous umbilical cord blood cells on CP children, there still needs to be a sufficient amount of data demonstrating the safety and efficacy before transplantation therapy can be used in other neonatal diseases. It is a priority to obtain standardized experimental models with quantitative functional endpoints and predictive clinical outcomes so that translational research can be implemented. In addition, it is important to address and modify the following for an effective future use of stem cell therapy: the route of delivery, cell dose, and timing of transplantation after diagnosis of neonatal brain injury. It should also be noted that because it is difficult to detect encephalopathy in premature babies due to the lack of advanced imaging devices and sufficient studies, initial cell therapy clinical trials will consist of full term infants. Because it is agreed that HIE involves several cell death pathways, it is expected that more laboratory research on combination therapies will be conducted in the future. Specifically, incorporating clinical stage therapies, such as hypothermia and other neuroprotective strategies, with stem cell transplantation therapy will be of future focus. Moreover, the safety and efficacy of these combinatorial strategies for neonatal hypoxic-ischemic brain injury can be maximized by following pertinent translational research guidelines [e.g., Ref. (32)].

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Could cord blood cell therapy reduce preterm brain injury?

Jingang Li*[1], *Courtney A. McDonald*[1], *Michael C. Fahey*[1,2], *Graham Jenkin*[1,3] *and Suzanne L. Miller*[1,3]

[1] *The Ritchie Centre, MIMR-PHI Institute, Clayton, VIC, Australia*
[2] *Department of Paediatrics, Monash University, Clayton, VIC, Australia*
[3] *Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia*

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Paulo Henrique Rosado De Castro, Universidade Federal do Rio de Janeiro, Brazil
Emily Camm, University of Cambridge, UK

****Correspondence:***
Suzanne L. Miller, Department of Obstetrics and Gynaecology, The Ritchie Centre, MIMR-PHI Institute, Monash University, Level 3, 27-31 Wright Street, Clayton, VIC 3168, Australia
e-mail: suzie.miller@monash.edu

Major advances in neonatal care have led to significant improvements in survival rates for preterm infants, but this occurs at a cost, with a strong causal link between preterm birth and neurological deficits, including cerebral palsy (CP). Indeed, in high-income countries, up to 50% of children with CP were born preterm. The pathways that link preterm birth and brain injury are complex and multifactorial, but it is clear that preterm birth is strongly associated with damage to the white matter of the developing brain. Nearly 90% of preterm infants who later develop spastic CP have evidence of periventricular white matter injury. There are currently no treatments targeted at protecting the immature preterm brain. Umbilical cord blood (UCB) contains a diverse mix of stem and progenitor cells, and is a particularly promising source of cells for clinical applications, due to ethical and practical advantages over other potential therapeutic cell types. Recent studies have documented the potential benefits of UCB cells in reducing brain injury, particularly in rodent models of term neonatal hypoxia–ischemia. These studies indicate that UCB cells act via anti-inflammatory and immuno-modulatory effects, and release neurotrophic growth factors to support the damaged and surrounding brain tissue. The etiology of brain injury in preterm-born infants is less well understood than in term infants, but likely results from episodes of hypoperfusion, hypoxia–ischemia, and/or inflammation over a developmental period of white matter vulnerability. This review will explore current knowledge about the neuroprotective actions of UCB cells and their potential to ameliorate preterm brain injury through neonatal cell administration. We will also discuss the characteristics of UCB-derived from preterm and term infants for use in clinical applications.

Keywords: preterm birth, low birth weight, brain damage, white matter injury, oligodendrocytes, cerebral palsy, umbilical cord blood, stem cells, hypoxia–ischemia, inflammation, periventricular leukomalacia

BACKGROUND

Impressive advances in perinatal and neonatal care have led to substantial improvements in survival rates for preterm infants born at <37 weeks gestation. However, survival of preterm infants may occur at a cost, with a strong causal link between preterm birth and subsequent neurological motor and cognitive deficits, including cerebral palsy (CP). In particular, more extremely preterm babies now survive than ever before with these infants at the greatest risks of short and long-term neurodevelopmental deficits (1). Despite the known etiological link between preterm birth and neuromotor and neuro-cognitive dysfunctions, there are currently no specific neuroprotective treatments available for preterm infants.

Stem, or stem-like cells, have drawn attention from scientists and the general public due to their potential to induce tissue repair and/or regeneration. Umbilical cord blood (UCB)-derived cells offer ethical and practical advantages over other stem-like cells given that collection can be obtained from the discarded placenta at birth. Such cells possess multiple proven [as per cord blood hematopoietic stem cells (HSCs)] and potential therapeutic uses, including recent evidence that UCB cells may mitigate newborn brain damage arising from term neonatal hypoxic-ischemic encephalopathy (HIE). However, despite the heightened neurological risks associated with preterm births, the potential use of UCB cells in preterm neonates has not yet been actively investigated. This article briefly describes the background, etiology, and pathophysiological mechanisms of brain injury in preterm infants, and summarizes current research on the use of UCB cells for therapeutic use in term and preterm perinatal brain injury. Potential implications for future clinical trials of UCB cell therapy in preterm infants are discussed.

PRETERM BIRTH AND CHILDHOOD NEUROLOGICAL DEFICITS

In 2010, 14.9 million babies worldwide were born preterm, accounting for approximately 11% of all births, with the rates and burden of preterm birth significantly increased in both low and high-income birth settings compared to the previous decade (2). Of all preterm births in the developed world, 16% are born before 32 weeks of gestation or weigh <1500 g (2), with this population of *very preterm* infants (born 28 to <32 weeks) or *extremely preterm* infants (<28 weeks) at the greatest risks for long-term physical and neurological morbidities. Indeed, in developed countries, preterm births account for 70% of neonatal deaths and up to 75% of neonatal morbidity (3), with the risks of death or disability profoundly increased in middle- or low-income birth settings, reflecting decreased resources for neonatal intensive care (4). In addition, in developed countries most preterm babies now survive

as a result of advances in neonatal intensive care such that the survival rate for extremely preterm infants is 90% (2).

Cerebral palsy is the most common physical disability of childhood, occurring in 2–2.5/1000 live births in developed countries. This rate is increased to approximately 90–100/1000 babies that were born at <32 weeks gestation (5, 6). Indeed, 35–50% of children with established CP were born preterm (7, 8). The major overt neurological manifestations of brain injury observed in children that were born preterm are spastic motor deficits, commonly accompanied by intellectual deficits. Less severe disturbances of motility, cognition, and behavior occur in 25–50% of survivors (9).

The economic cost of preterm birth and CP are high due to the need for neonatal intensive care and ongoing long-term complex health care. The National Institute of Medicine estimated that the lifetime cost of all preterm births is $26.2 billion USD per year in the USA (10). The financial burden of CP in the USA has been separately costed and estimated at $11.5 billion USD (11) and is indicative of the large financial burden association with preterm birth and CP. This is in addition to the significant burden placed on families and society who care for children and adults with CP. There is therefore an enormous demand to prevent or reduce brain injury in preterm infants, to reduce the subsequent neurodevelopmental sequelae, and consequently decreasing the large socio-economical burden.

The complications associated with preterm birth and brain injury are complex and involve multiple overlapping adverse pathways, but it is clear that preterm birth is strongly associated with damage to the white matter of the immature brain. Therefore, an understanding of white matter injury (WMI) is a critical component required for the treatment of preterm brain injury.

WHITE MATTER INJURY

Fetal brain maturation and functional development involves a series of organizational processes including neurogenesis, cell migration, cell differentiation, synaptogenesis, and axonal myelination. The development of white matter requires mature oligodendrocyte glial cells to produce myelin and ensheath the axons of neurons, and thus oligodendrocytes play a crucial role in fast signal transmission along neurons and throughout the brain. Injury to these cells impairs, usually irreversibly, myelination. Oligodendrocytes develop according to a well-defined lineage. Pre-oligodendrocytes are the predominating oligodendroglial cell at gestational age 24–32 weeks in humans. They are exquisitely vulnerable to pro-inflammatory cytokines, excitotoxicity, oxygen free radical attack, and hypoxic stress, and rapidly undergo apoptosis under adverse conditions (12–15). It is believed that this selective vulnerability of the pre-oligodendrocytes in preterm infants restricts the number and functional ability of mature oligodendrocytes to undergo the process of laying down of white matter and formation of myelin fibers, thus causing very preterm and extremely preterm infants to be most susceptible to WMI (9, 16, 17). Thus, preserving oligodendrocytes and their precursor cells is fundamental to reducing injury to the developing white matter of the brain. Most commonly, preterm brain injury is evident in the periventricular white matter adjacent to the lateral ventricles, so-called periventricular leukomalacia (PVL). WMI is detectable in at least 50% of infants born very preterm or extremely preterm, and is a strong indicator of long-term neurological adverse outcome. Nearly 90% of preterm infants who later develop spastic CP have evidence of WMI (9). Half of the children identified as having WMI will have cognitive and/or behavioral and/or attention deficits (18–20). Clinical imaging studies demonstrate that myelin loss (hypomyelination) and disorganization of major white matter fiber tracts correlate with functional impairments in children with CP and PVL (21, 22).

Pathologically, WMI is a condition demonstrated by coagulation and necrosis of white matter near the lateral ventricles, accompanied by gliosis (23). The periventricular area is vulnerable to ischemia in the preterm brain, which, in part, is anatomically due to poor vascularization and immature cerebrovascular autoregulation (24, 25). This may result in focal PVL. But, in many cases, WMI is widespread and incorporates periventricular, subcortical, and callosal white matter, as well as the internal capsule. WMI, and in particular PVL, has two distinct histopathological appearances, described as either cystic or non-cystic (diffuse) WMI. Cystic WMI typically affects all types of cells and is therefore considered the more severe type and is closely linked with CP, whereas diffuse PVL mainly targets pre-oligodendrocytes and is considered less severe but nonetheless is linked to cognitive and behavioral impairments, and CP (9). It is generally considered that the gray matter is not as susceptible to preterm insults as is white matter, but the pre-oligodendrocytes also present in gray matter are not spared, leading to damage involving the cerebral cortex, thalamus, and basal ganglia (26, 27).

There is a growing understanding of the etiology of preterm brain injury, likely involving one or more interactions between fundamental immaturity of the brain, vulnerability of white matter developmental processes, and the adverse effects of two principal upstream insults: hypoxia–ischemia and infection/inflammation. Hypoxia–ischemia and infection/inflammation are relatively common in the preterm period, and have profound adverse effects on white matter development (28–30).

HYPOXIA–ISCHEMIA AND WHITE MATTER INJURY

After an hypoxic-ischemic insult, microglia and macrophages within the brain's white matter exhibit immunoreactivity for interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and infiltrate to lesion sites. Astrocytes become hypertrophied and diffuse gliosis is evident within 24 h. Loss of oligodendroglial lineage cells and impairment of myelinogenesis is evident within 10 days following hypoxia–ischemia (9, 31–35). When the insult has been prolonged or severe, brain injury is exacerbated through influx of cytokines and chemokines via the damaged blood–brain-barrier (BBB), thereby further increasing inflammatory mediators within the brain (36).

In response to a significant hypoxic-ischemic insult, secondary pathways of injury are also initiated and evolve over days. These adverse pathways include mitochondrial dysfunction, excitotoxicity, apoptosis, oxidative stress, and initiation of additional inflammatory processes (37). A further adverse effect of hypoxia–ischemia is the disruption of normal growth and differentiation factors driving brain development, decreasing concentrations of signaling proteins and nutrients that include neurotrophic factors vital for inhibition of programed cell death (38, 39). For example,

brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 play important roles in promoting neuronal growth and differentiation, connective plasticity, and neuronal survival through their interaction with tyrosine kinase β-receptors, but these are each affected in the preterm brain in response to hypoxia–ischemia (40). Additional neuron and glial cell loss occurs over days and weeks after a sentinel insult, resulting from chronic deprivation of neurotrophic factors, decreased synaptic input from neighboring cells, and loss or recruitment failure of local neural and glial stem and progenitor cells (41, 42). The severity and duration of neurotrophic factor deprivation directly correlates to long-term neurological outcome (38, 42).

In addition, activated astrocytes and microglia mediate the release of reactive oxygen species (ROS) and reactive nitrogen species (RNS), leading to increased protein nitration and oxidative stress in response to hypoxia–ischemia and brain inflammation (43). In the context of preterm birth, a compromised intrauterine environment may induce excess release of free radicals and, combined with the transition to an extra-uterine high oxygen environment, may overwhelm endogenous antioxidant enzymes, resulting in preferential death of pre-oligodendrocytes and contributing to the development of WMI within the preterm brain (43–46).

INFECTION/INFLAMMATION AND WHITE MATTER INJURY

Fetal and neonatal exposure to infection and/or inflammation is recognized as a principal contributor to preterm birth and WMI. Maternal intrauterine infection including chorioamnionitis is associated with increased levels of pro-inflammatory cytokines (IL-6, IL-8, TNF-α, and IL-1b) in the amniotic fluid and cord blood (47–49) and is one of the most important causes of preterm birth <30 weeks of gestation (50). Maternal intrauterine infection presents a significant risk for WMI and CP (51, 52). Neonatal sepsis is also a risk factor for WMI in infants that were born preterm (53, 54).

Adverse inflammatory stimuli during fetal or neonatal life induce a systemic and central nervous system (CNS) response via activation of innate and adaptive immune systems. Microglia are the primary mediators of the brain's immune response, mediating the pro- and anti-inflammatory response to remove pathogens, via binding of toll-like receptors (TLRs) with ligands, pathogen-associated molecular patterns (PAMPs), and/or danger-associated molecular patterns (DAMPs) (55, 56). However, prolonged microglial activation can cause brain injury (55). Microglia are at their peak density in white matter during the WMI-vulnerable period (57), making them fundamental in producing WMI (44). Lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria and a form of PAMP, and binds receptors including TLR-4 and CD14 on microglia, initiating a signal transduction cascade that ultimately activates transcription factors such as nuclear factor-kappaB (NF-κB). In turn, this leads to up-regulation of cytokines, chemokines, and complement proteins, and over time this response can sensitize the developing brain to secondary insults thereby contributing to sustained CNS inflammation. Cytokines may directly act upon oligodendrocytes to induce cell death, as evidenced by *in vitro* studies on human oligodendrocytes where TNF-α and interferon-γ (IFN-γ) induced dose-dependent cell necrosis (58). *In vivo* administration of high-dose LPS to preterm fetal sheep results in significant cerebral hemodynamic changes that cause cerebral ischemia and PVL-like fetal brain injury (59), while low-dose LPS, insufficient to cause fetal hypoxia, induces diffuse WMI and microglial invasion, where the degree of microglial activation is correlated to the presence of WMI (60).

The downstream pathways that result from hypoxia–ischemia, or inflammatory stimuli, are complex and are not mutually exclusive. Hypoxia–ischemia and increased ROS are known to induce an inflammatory reaction and, conversely, pro-inflammatory mediators lead to the generation of free radicals and oxidative stress. This interaction is driven by NF-κB. In normal-state resting cells, the NF-κB protein complex remains within the cytoplasm, bound to inhibitory IκB protein. Pro-inflammatory cytokines, LPS and viruses cause proteolysis of IκB, allowing dissociation from NF-κB, and the nuclear translocation of NF-κB where it activates gene transcription (61). Additionally, tissue hypoxia and oxidative stress can modulate NF-κB release (61). Thus, hypoxia–ischemia can induce inflammation via microglial activation, and conversely infection/inflammation can induce hypoxia–ischemia through hypotension (62). Indeed, preterm infants have been shown to have higher risk of WMI when chorioamnionitis and placental perfusion deficits are present together (63).

LIMITATIONS OF CURRENT TREATMENTS OF WHITE MATTER INJURY

Although there have been major clinical and scientific advances in neonatal care over the last decade, currently only antenatal corticosteroid are proven to reduce the risk of intraventricular hemorrhage (IVH) (64). Other strategies in preterm infants, such as use of erythropoietin, melatonin, indomethacin, antenatal magnesium sulfate, therapeutic hypothermia, or delayed cord clamping remain at the experimental investigation stage and are not of proven benefit (65, 66). Thus, current management for preterm brain injury has, until now, been restricted to supportive strategies.

One of the biggest hurdles for identifying neuroprotective strategies for preterm infants is the multi-faceted etiology of the brain damage. As described in the section above, the primary antenatal causal factors that may induce brain injury include maternal/fetal infection and/or chronic placental perfusion insufficiency (67, 68). Postnatal factors may exacerbate or cause brain injury, including repetitive subacute/chronic hypoxia–ischemia due to poor lung function and ventilation, and free radical imbalance following oxygen reperfusion in response to a high oxygen extra-uterine environment or oxygen administration (44, 46, 69). Neonatal chronic cerebral hypoperfusion, hypotension, hypocarbia, or symptomatic persistent ductus arteriosus (70, 71), IVH with or without post-hemorrhagic ischemia or hydrocephalus (72), infection (53, 54), hypoglycemia, and glucocorticoid administration are also involved in the progression of brain injury (68, 73). Moreover, even preterm birth without exacerbating factors can result in subtle white matter pathology (69). Thus, it can be appreciated that unlike term neonatal HIE, these insults do not necessarily occur around the time of delivery, and it may therefore be difficult to recognize the timing of the onset of a sentinel (or

exacerbating) insult. It would therefore be likely that preterm brain injury would be best treated with a therapy, or therapies, with multiple neuroprotective mechanisms and with a long therapeutic window. Any therapy should be targeted at WMI as the predominant neuropathology. Additionally, such a treatment would be aimed at one or more of the following – reducing inflammation and free radical attack, halting the progression of cell death programing, and/or replacing damaged oligodendrocytes in order to remodel areas of WMI and normalize myelination. We will present data to support the therapeutic potential of neonatally administered UCB-derived cells, for protection and repair of the preterm brain.

STEM CELLS

Stem cells are characterized by their ability to undergo self-renewal and to differentiate into multiple cell types. In general, stem cells can be classified into three major categories on the basis of their source, namely embryonic stem cells (ESCs), fetal-derived stem cells, and adult stem cells. ESCs are pluripotent, are able to generate cells from all three germ layers and can be maintained in culture indefinitely (74), providing a limitless source of precursor cells for the regeneration of damaged tissue. However, due to the pluripotent nature of ESCs they are also tumorigenic and transplantation of these cells currently presents significant safety concerns (75), and has cautioned their use in clinical trials. ESCs are also obtained from embryos, presenting ethical issues. With recent advances, it has become possible to reprogram somatic cells and generate induced pluripotent stem (iPS) cells (76). These cells may be able to overcome some of the limitations of ESCs, i.e., ethical issues, and enable the generation of patient-specific iPS cell lines. However, they are also tumorigenic (77), and this issue remains unresolved. Adult stem cells, or stem-like cells, include mesenchymal stromal cells (MSCs), that can be obtained from a number of sources including bone marrow, adipose tissue, and dental pulp, and neural progenitor cells (NPCs) that are found in the subventricular zone of the brain and comprise multipotent stem/progenitor cells that can differentiate down the neural lineage, including to neurons and glial cells (78). NPCs can be isolated from the adult brain and expanded for several passages whilst retaining their undifferentiated state. Lastly, fetal-derived stem-like cells can be obtained from placental tissue (79) and UCB (80) and include placental and umbilical cord MSCs (UC-MSCs), amnion epithelial cells (AECs), HSCs, and endothelial progenitor cells (EPCs) (81). Given these cells are isolated from fetal tissue, they tend toward greater differentiation and expansion potential than adult stem cells. Fetal-derived stem cells can be easily isolated from tissue that is routinely discarded at birth, they are abundant due to the large number of births each year, and their collection raises no ethical concerns.

Stem-like cells sourced from placenta and umbilical cord (UC) have been studied pre-clinically for treatment of a variety of diseases including multiple sclerosis (82, 83), stroke (84, 85), bronchopulmonary dysplasia (86, 87), and CP (88, 89) and may be beneficial for reducing disease burden in these conditions. For MSCs alone, there are currently >400 clinical trials listed on clinical trials.gov (search: "mesenchymal stem cell"). However, there are numerous clinics around the world that are already capitalizing on the *promise* of stem cell treatment and are offering stem cell therapies for financial gain to families of those with conditions including CP. This has been coined stem cell tourism (90). It is therefore imperative that well-planned and controlled pre-clinical and clinical trials are conducted to establish the safety, short-, and long-term efficacy, and mechanisms by which stem cell therapies may provide benefit, which in turn will enable treating clinicians and patients to make informed decisions regarding the use of stem cell treatments.

UMBILICAL CORD BLOOD

Umbilical cord blood is a rich source of HSCs, accounting for 0.5–1.0% of mononuclear cells (MNCs) in term UCB (91), used to treat patients with abnormal hematopoietic conditions, childhood leukemia, or metabolic diseases (92). HSCs are positive for CD34 and CD45, and defined by their capacity to self-renew and give rise to multiple blood lineages. Traditionally, bone marrow-derived HSCs were used to treat these conditions, however, UCB is easier to obtain, less expensive and less likely to trigger a deleterious immune response or rejection in the recipient (93). HSCs from human UCB (hUCB) are also more primitive than bone marrow-derived HSCs, have longer telomeres, have a higher colony-forming capacity and can repopulate blood lineages over a long period of time (94, 95). Given these advantages, more than 3000 hUCB transplants are now performed each year for blood and other disorders (96). Other strong advantages for the use of UCB for transplants include that it can be tissue typed, screened for viral biomarkers, processed and banked, allowing the supply for both urgent and directed transplants (97) and the volume and number of cells that can be attained is generally very good. In term births, a large volume of UCB can be collected [38–42 weeks: 102 ± 30 ml, containing $11.3 \pm 6.2 \times 10^8$ total nucleated cell (TNC)] (91). However, in preterm birth, or in pregnancy complications [such as intrauterine growth restriction (IUGR)], there is reduced UCB volume for collection [34–37 weeks: 90 ± 32 ml, $7.7 \pm 4.8 \times 10^8$ TNC; 25–33 weeks: 62 ± 31 ml, 3.3 ± 3.5 TNC] (91), which is problematic if UCB collection is required or requested. Furthermore, it is not known how antenatal complications, such as IUGR or chorioamnionitis, may change the composition of the stem and stem-like cells present in the UCB, and whether differences in cell composition may impact its therapeutic utility.

Umbilical cord blood is not only a useful source of HSCs, but also contains a number of other stem/progenitor cell types including MSCs and EPCs (80). Moreover, UCB is a rich source of immunosupressive cells, such as regulatory T cells (Tregs) (98). MSCs are multipotent adult progenitor cells that have a broad potential for repair of injured tissue. MSCs are characterized by their morphology, phenotype, and differentiation potential to form osteoblasts, chondrocytes, and adipocytes (82). MSCs are a plastic adherent cell population with the absence of CD34, CD45, and CD133, and are positive for CD13, CD29, CD44, CD73, and CD90 (80, 99). MSCs can indeed be isolated from UCB, but at a very low frequency and cellular fraction, with success rates for isolation ranging from 40 to 60% (99, 100) and, in one study, only 8% of UCB units could be effectively expanded into MSC-like colonies (100). While the frequency of MSCs is low in UCB (0.002% of MNCs in term UCB) (101), UCB–MSCs show a strong

proliferation capacity and can be maintained longer in culture than MSCs derived from other sources (99). Furthermore, following exposure to neural differentiation factors, hUCB–MSCs express a number of neural cell antigens, including glial fibrillary acidic protein (found in astrocytes), TuJ-1 (neural progenitor), vimentin, and nestin (102).

Endothelial progenitor cells, isolated from bone marrow, peripheral blood, and UCB can be differentiated into mature endothelial cells *in vitro* and, in animal models of ischemia, can incorporate into sites of active angiogenesis to stabilize and promote the growth of new blood vessels (103). While EPC classification remains contentious, they are generally characterized by the expression of CD133, CD34, and vascular endothelial growth factor (VEGF) receptor-2 (104). EPCs are estimated to make up 1–2% of HSC-containing CD34+ cell fraction in term UCB, representing 1 in 10^7 MNCs (105). Despite the low number, EPCs isolated from hUCB have been shown to have a stable endothelial phenotype and a higher proliferative capacity compared to those isolated from peripheral blood, making UCB a superior source for the isolation of EPCs. Studies are now being conducted to optimize the isolation of the three major cell types (EPCs, HSCs, and MSCs) from single UCB units (80).

Regulatory T cells should also be considered as potential useful cells to be isolated from UCB. Tregs are immunosupressive T cells that can maintain self-tolerance, prevent autoimmunity, inhibit rejection of transplants, and regulate the immune response to infectious disease (106). Tregs isolated from UCB exhibit a predominantly naïve phenotype, which is associated with a significantly enhanced proliferative potential compared to adult Tregs (107). It has been suggested that the low incidence of graft-versus-host-disease (GVHD) associated with UCB transplants is due to the presence of Tregs (108), adding to their importance for UCB transplants and their potential utility for treatment of inflammatory conditions.

Optimal selection of UCB units for HSC transplants includes determination of TNC content, CD34+ cell count, and HLA and blood group matching of the recipient and donor (97). However, there is sparse information related to other cells of interest within UCB. Given the increasing likelihood that children born preterm may request autologous UCB cell collection and treatment for brain injury (see below), it is imperative that we investigate the similarities and differences between term and preterm UCB. This knowledge will inform the design of clinical trials that will decide whether autologous or allogeneic UCB transplants will be best placed to treat neurological impairments in preterm-born infants. To date, it has been shown that the frequency of CD34+ cells in preterm neonates was twofold increased compared to those in term neonates (109) and, for a given gestational age, each 500 g increase in birth weight contributed to a 28% increase in CD34+ cell counts (110). In preterm infants, the immunophenotypic profile of UCB–CD34+ cells shows a significantly higher expression of CD33, and a lower expression of CD38, CD117, and HLA-DR, indicating preterm UCB has a higher percentage of primitive CD34+ subsets, while term UCB has a higher percentage of committed cells (111, 112). With specific regard to EPCs, preterm (28–34 weeks) UCB units have a fourfold increase in endothelial colony-forming cells compared to term UCB (113). However in compromised placental conditions such as preeclampsia, EPCs are decreased in term UCB, and not different to those in preterm UCB (114). Similar to other cell types, MSC population is also richer in preterm UCB compared with term, with a significant inverse correlation between the gestational age and presence of MSCs (101, 115). Furthermore, studies to date have predominantly assessed cell number and not cell function over gestation, where functionality may be a more important marker of efficacy than absolute cell number. As has been shown with hAECs, while preterm cells have a high proliferative capacity, they are functionally immature and cannot differentiate into other cell types (116). The presence of Tregs has also been assessed over gestation, and is reportedly increased in preterm UCB compared to term UCB (107), but Tregs from preterm UCB secrete significantly less IFN-γ (117). Furthermore, Tregs in UCB from IUGR infants at term were decreased compared to those in UCB units from appropriately grown babies (118).

Most studies to date examining perinatal brain injury have utilized UCB–MNCs (**Table 1**), but UCB–MNCs is composed of a variety of cells of interest including immature T cells, B-cells, monocytes, and stem-like cells including HSCs, EPCs, and MSCs. The fraction or combination of UCB cells responsible for neural repair remains to be established. UCB–MSCs have attracted interest for some time because of their multilineage differentiation potential, strong capacity for immune modulation, and low immunogenicity. Indeed, expanded hUCB–MSC transplantation has shown promise in protecting against perinatal brain injury in pre-clinical animal studies (119–121). Despite this, the clinical application of purified UCB–MSCs is currently limited by their low numbers and low success rate for isolation. On the other hand, CD34+ cells have been shown to reduce brain injury in neonatal hypoxic-ischemic mice, with a transient augmentation of cerebral blood flow in the peri-infarct area (122). UCB–CD133+ cells, the fraction enriched for EPCs and HSCs, also reduces infarct volume in a rat model of stroke (123).

In addition to stem-like cells, other cellular fractions in UCB have also been shown to have potentially important neuroprotective roles. When hUCB–MNCs was depleted for CD14+ monocytes, there was no decrease observed in microglial activation or functional recovery following administration (124), suggesting that monocytes are essential for mediating the neuroprotective benefits of hUCB cells in hypoxic-ischemic rats. In addition, a further study showed that a single injection of hUCB-derived T cells (CD4+) induced endogenous NPC proliferation for 2 weeks and promoted increased neuronal cell survival in rats (125). The therapeutic effects of stem cells are now thought to be independent of tissue engraftment (89, 126–129), although many studies have shown that transplanted UCB cells can migrate selectively toward ischemic areas of damaged brain (127, 130). It is widely considered that regenerative effects of stem cells are principally derived from indirect paracrine and trophic effects, and increasing the regenerative capacity of the brain, rather than via direct cell replacement (38, 128, 129, 131, 132). However, it is important to note that the studies referred to above have utilized hUCB in a xenogeneic setting. As such, the ability of the transplanted cells to survive and differentiate may be compromised (133). To our knowledge, the ability of autologous UCB cells to home to the site of injury and differentiate into neurons or neuroglial cells has not

Table 1 | Outcome of umbilical cord blood interventions in neonatal hypoxia–ischemia.

Cell type	Animal model	Administration			Engraftment		Histology assessments		Functional assessments		Other	Reference
	Injury type	Timing	Dose	Route	Days	Results	Days	Outcomes	Days	Outcomes		
hUCB–MNCs	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IP	21 days	Many cells in ischemic hemisphere. No sign of transdifferentiation	NA	NA	21 days	Alleviation of spastic paresis		Meier 2006 (127)
	P7 rats, HI 120 min	24 h after HI	1×10^7 cells	IV jugular	21 days	Few cells in brain tissue	21 days	No change in volume of injured hemisphere	21 days	No change on spatial memory deficit		de Paula 2009 (192)
	P7 rats, HI 90 min	3 h after HI	2×10^6 cells	IP	2 days	Few cells in ischemic cortex and striatum	2 days	Decreased neuronal death in striatum, and microglial activation in cortex	4, 7 days	Improved developmental sensorimotor reflexes only at 4 days		Pimentel-Coelho 2010 (134)
	P7 rats, HI 150min	2–3 h after HI	1.5×10^4 cells (± mannitol)	IV jugular	14 days	Few cells in ischemic hippocampus	NA	NA	7, 14 days	20–25% improvement in rotarod and elevated body swing tests	Increased growth factors in brain, CA1 dendrites	Yasuhara 2010 (138)
	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IP	42 days	Many cells in peri-infarct area	42 days	No change in size of hemispheric lesion	42 days	Improved sensorimotor function, cortical maps, and receptive fields, and reduced hyperexcitability		Geissler 2011 (135)
	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IT	14 days	hUCB cells were localized in astrocyte-rich zone	2, 14, and 44 days	Decreased activation of microglia/macrophages and reactive astrogliosis, and reduced peri-lesional astrocytic wall	14, 44 days	Improved motor function (forelimb use bias, muscle strength and distal spasticity) both short- and long-term	Downregulation of Connexin 43	Wasielewski 2012 (136)
	P7 rats, HI 80 min	24 h after HI	1×10^7 cells	IP	NA	NA	2, 14 days	Decreased lesion-induced apoptosis, increased neurons	NA	NA	Increased the expression of proteins Tie-2, occludin, BDNF and VEGF in the lesioned brain	Rosenkranz 2012 (141)
	P7 rats, HI 120 min	2 h after HI	1×10^6, 1×10^7, 1×10^8 cells	IV jugular	7 days	Cells in the cortex and the hippocampus	8 weeks	No change in low-dose group. Decreased brain atrophy in medium- and high-dose groups	8 weeks	Cognitive improvement at the highest dose only		de Paula 2012 (133)
	P7 rats, HI 90 min	24 h after HI	1×10^7 cells	IV jugular	1, 3, and 10 weeks	Many cells were in ischemic periventricular region at 1 week, but very few at 3 and 10 weeks	10 weeks	No decrease in tissue loss volume, decreased neuronal loss in neocortex	10 weeks	Improved performance in a battery of behavioral tests		Bae 2012 (139)
	P7 rats, HI 120 min (+cyclosporin A)	24 h after HI	3×10^6 cells	IVen	NA	NA	24, 72 h, 7, 14 days	Decreased neuronal loss in cortex and CA1 of the hippocampus	NA	NA	Increased Shh and Gli1 protein levels	Wang 2014 (137)
hUCB–CD34+	P12 SCID mice, MCAO	48 h after HI	1×10^5 cells	IV femoral	24 h, 10 days	Few cells at 24 h, very few at 10 days	7 weeks	Decreased brain atrophy	9 days, 7 weeks	No effect on rotarod or open-field tests	Transient augmentation of CBF in peri-infarct area	Tsuji 2014 (122)

(Continued)

Table 1 | Continued

Cell type	Animal model	Administration			Engraftment		Histology assessments		Functional assessments		Other	Reference
	Injury type	Timing	Dose	Route	Days	Results	Days	Outcomes	Days	Outcomes		
hUCB–MSCs (passage 10)	P7 rats, HI 150 min (+cyclosporin A)	3 days after HI	1×10^5 cells	Intra-cerebral	7 days	Differentiation into astrocytes but not neurons	28 days	Reduced cortical neuronal loss	14, 21, 28 days	Improved neurological score		Xia 2010 (119)
(passage 5)	P10 rats, MCAO	6 h after HI	1×10^5 cells	IVen	NA	NA	28 days	Decreased apoptosis, microglial activation and astrogliosis in penumbra	28 days	Functional improvements (rotarod and cylinder test). GM1 enhanced the behavioral recovery	Improved survival and body weight gain, decreased lesion volume on MRI	Kim 2012 (120)
hUC-MSCs (passage 3)	P7 rats, HI 120 min (+cytarabine)	24 or 72 h after HI	5×10^6 cells (± GM1)	IP or IV jugular	35 days	More cells were in ischemic frontal cortex after iv than ip, with neural differentiation around infarct region	35 days	Decreased gliosis in ischemic regions	7 days, 20 days, 4 weeks	More improved locomotor function in animals given cells at 24 h than 72 h		Zhang 2014 (130)

BDNF, brain-derived neurotrophic factor; CA1, cornu ammonis 1 of the hippocampus; CBF, cerebral blood flow; GM1, ganglioside; HI, hypoxic–ischemia (unilateral ligation of the carotid artery followed by 8% oxygen systemic hypoxia); hUCB–MNCs, human umbilical cord-mononuclear cells; IP, intraperitoneal; IT, intrathecal; IV, intravenous; IVen, intraventricular; MCAO, middle cerebral artery occlusion; MSCs, mesenchymal cells; NA, not applicable; P, postnatal day; UC, umbilical cord; VEGF, vascular endothelial growth factor.

been investigated. Thus, the engraftment potential of UCB cells remains poorly characterized, and requires further investigation in studies using autologous transplantation.

NEUROPROTECTIVE PROPERTIES OF UMBILICAL CORD BLOOD

A number of studies have demonstrated significant and reproducible neuroprotective effects in rodent models of term neonatal hypoxia–ischemia using UCB–MNCs (127, 133–139), UCB–MSCs (119, 120), or UCB–CD34+ cells (122). Meier and colleagues first showed that intra-peritoneal administration of hUCB–MNCs alleviated spastic paresis in the Rice–Vannucci model of neonatal hypoxic-ischemic rats (127, 140). Following this, other rodent studies have shown that hUCB–MNCs induce significant improvements in sensorimotor performance (134–136, 138) and reduction in neuronal loss (133, 134, 137–139, 141). Recent studies also showed long-lasting neuroprotective effects of hUCB–MNCs in behavioral and cognitive outcomes at 8 and 10 weeks after ischemic insult (133, 139), with decreased brain atrophy (133). Animals treated with intra-cerebral hUCB–MSCs also demonstrated improved neurological function and tissue repair (119, 120).

From pre-clinical results obtained to date, we hypothesize that UCB cells may act in a neuroprotective manner via diverse actions, including anti-inflammatory effects, immunomodulation, and neurotrophic growth factor release to promote endogenous neurogenesis.

ANTI-INFLAMMATORY AND IMMUNO-MODULATORY ACTIONS OF UMBILICAL CORD BLOOD

A principal mechanism whereby UCB cells regulate neurological repair is via anti-inflammatory actions. UCB administration can dampen the expression of pro-inflammatory cytokines (IL-1α, IL-6, IL-1β, and TNF-α), enhance anti-inflammatory cytokines (IL-10), secrete chemotactic proteins (monocyte chemotactic protein 1), and modulate immune macrophage and T cell function (142, 143). As described above, hypoxia–ischemia induces an acute brain inflammatory response with activation of microglia and macrophages and reactive astrogliosis associated with peri-lesional up-regulation of connexin 43, the major astrocytic gap junction protein (144). Administration of hUCB–MNCs normalizes inflammatory balance, reduces microgliosis and astrogliosis (134, 136), and down-regulates connexin 43, which in turn restores BBB function to moderate inflammatory cell influx into the brain (136).

NEUROTROPHIC FACTOR ACTIONS OF UMBILICAL CORD BLOOD

Transplanted UCB–MNCs or MSCs reportedly enhance neurological recovery via secretion of a wide variety of trophic factors including BDNF, glial cell line-derived neurotrophic factor (GDNF), nerve growth factors NT-3 and NT-5, angiogenin, VEGF, fibroblast growth factor-2, and epidermal growth factor. Together, these act to promote endogenous neuronal growth and neurogenesis, angiogenesis, encourage remyelination, and synaptic connections, and decrease cellular apoptosis (141, 145–147). Transplantation of hUCB is associated with reduced levels of cleaved-caspase-3 protein in hypoxic-ischemic newborn rats, indicative of reduced apoptosis, with BDNF identified as playing

a role in inhibition of apoptosis and inflammation (141). Further, hUCB cell administration after hypoxia–ischemia increases expression of Tie-2 and occludin proteins, and increases expression of VEGF, indicating that UCB transplantation may increase endogenous angiogenesis and improved BBB integrity within the damaged brain. *In vivo*, MSCs provide trophic neuroprotection following injury by secreting physical tissue scaffold to surrounding tissues, while UCB–CD34+ cell transplantation enhances functional recovery and reduces both infarction and apoptosis in a rat model of spinal cord injury, mediated by the production of both VEGF and GDNF (148). UCB-derived CD133+ cells promote a threefold improvement in axonal regrowth, a 35% reduction in apoptosis and vascular and neuronal protection following hypoxia on organ co-culture of brain motor cortex cells and spinal cord from postnatal day 3 rats, suggesting that the trophic effects from CD133+ cells contributes to neuroprotection (149).

Hypoxia–ischemia stimulates endogenous proliferation of NPCs (150). However, if an insult is severe, brain damage still occurs, and the restorative proliferation of NPCs may be ameliorated, with activated NPCs failing to survive to mature neurons (150, 151) or differentiating into astrocytes (152). Recently UCB–MNC transplantation has been shown to promote the proliferation of endogenous NPCs, and reduce glial differentiation, an action mediated via the Sonic Hedgehog signaling pathway, resulting in the alleviation of brain injury in hypoxic-ischemic neonatal rats (137). These results support the ability of UCB cells to respond to insult with paracrine and trophic actions, initiating a regenerative environment mediated by resident cell populations within the brain.

PRETERM BRAIN INJURY ANIMAL MODELS AND CELL THERAPY

The majority of experimental studies described above that have investigated the neuroprotective actions of hUCB have been undertaken in rodent models of neonatal (term) hypoxic-ischemic brain injury (119, 120, 122, 127, 133–139). Injury to the human brain at the time of term birth induced by hypoxia–ischemia predominantly causes deep gray matter neuronal injury within the basal ganglia and hippocampus, together with injury to neighboring white matter – this is appropriately reflected in rodent and large animal studies of term hypoxia–ischemia. However, this distribution of injury is quite different in preterm WMI, reflecting susceptibility and region-specific effects following hypoxia–ischemia and other insults as brain maturation progresses.

Animal models exploring the injury profile and mechanisms of preterm pre-oligodendrocyte and WMI, have utilized either an hypoxic-ischemic insult, or exposure to LPS-induced inflammation. Excitotoxicity models such as administration of excitatory amino acid agonists quinolinic acid and ibotenate have also been used (26, 153). It is important to note that preterm infants mostly suffer hypoxic-ischemic insults that are subacute or chronic, in contrast to term infants where HIE is principally due to an acute severe insult (154). Irrespective of which experimental insult is utilized, the maturational age of the CNS is critical (26). In addition, the choice of species is important. It is well described that induction of predominant WMI is problematic in rats and mice due to the different CNS anatomy of rodents that, besides being non-gyrencephalic and having a different vascular anatomy, demonstrates a much lower white/gray matter ratio than in humans. In contrast, the pattern of WMI in rabbits, cats, dogs, and sheep has a distribution and morphological appearance closer to that of human preterm brain injury induced by either hypoxia–ischemia or LPS administration (26, 59). The fetal rabbit brain myelinates with a similar perinatal time course to the human, and maturation of oligodendrocytes begins antenatally (155). *In utero*, hypoxia–ischemia to the preterm rabbit fetus causes postnatal hypertonic motor deficits that resembles CP, making rabbits a very good model for postnatal behavioral studies (156). Many further studies have been undertaken utilizing fetal sheep models of preterm WMI because of their abundance of cerebral white matter, their anatomic similarities to the preterm infant, and an ability to monitor the systemic and brain response to insult (37, 59, 157). However, because of cost, availability of antibodies/sequence data and genetically modified animals, rodent WMI studies are valued as complementary models (26). To date, only hAECs have been examined in a non-rodent (fetal sheep) model of preterm WMI. Yawno and colleagues demonstrated that administration of hAECs suppressed the up-regulation of activated microglia, and reduced gray and WMI in response to LPS in preterm fetal sheep (88) (**Table 2**).

PRETERM BRAIN INJURY AND UMBILICAL CORD BLOOD

Hall and colleagues demonstrated that in postnatal day 2 rats, intravenous hUCB–MNC administration preserves white matter structures following an hypoxic-ischemic insult. This timeframe corresponds to the period of white matter vulnerability in human preterm infants between 24 and 30 weeks of gestational age (158). Specifically, IV infusion of hUCB–MNCs at 48 h post-ischemia reduced WMI based on quantification of myelin basic protein. A direct protective effect of UCB–MNCs on oligodendrocyte injury induced by oxygen/glucose deprivation (OGD), which produces hypoxic-ischemic-like injury *in vitro*, was also identified (158). Although the data are limited, it appears that hUCB–MNCs have therapeutic potential for the protection of oligodendrocytes and thereby prevention of WMI in a premature rat model of ischemia.

ANTIEPILEPTIC EFFECTS OF UMBILICAL CORD BLOOD

The incidence of seizures in very low birth weight infants is 5.6%, while the occurrence in those infants identified as having PVL is 18.7% (159, 160). Seizures are typically observed in more severe cases of PVL and those born at lower gestational ages and birth weights (159, 160). Recent studies demonstrate the antiepileptic actions of hUCB–MNCs. Transplantation of hUCB–MNCs 90 min after the onset of status epilepticus in rats, induced by lithium and pilocarpine chloride, protected against neuronal loss in the hippocampus for up to 300 days. Additionally, MNC-transplanted rats had reduced frequency and duration of recurrent seizures, suggesting early administration could protect against the establishment of epilepsy (161). Furthermore in a single case of an infant with infantile spasms (West syndrome) and X-linked T/B + NK-severe combined immunodeficiency, allogeneic UCB transplantation together with topiramate and immune-modulating agents (corticosteroids, intravenous immunoglobulin,

Table 2 | Outcome of cell-based interventions in preterm brain injury.

Cell type	Animal model	Administration			Engraftment		Histology assessments		Functional assessments		Others	Reference
	Injury type	Timing	Dose	Route	Days	Results	Days	Outcomes	Days	Outcomes		
hUCB–MNCs	P2 rats, MCAO	48 h after stroke	1×10^6 cells	IV	NA	NA	4 days	Reduced white matter damage	NA	NA	UCB–MNCs directly reduced apoptosis of oligodendrocytes cultured under oxygen glucose deprivation *in vitro*	Hall 2008 (158)
hUCB–MNCs	P5 rats, excitotoxicity (ibotenate)	within 6 h or 24 h after injection	1×10^6 or 10^7 cells	IP or IV	5 days	No cells detected	5 days	No changes in lesion size, microglial activation, astrogliosis, or cell proliferation. Increased white matter damage with increased microglial activation by ip administration	NA	NA		Dalous 2013 (89)
hUC-MSCs (Passage 3)	P3 rats, HI 240 min	0, 1, 2 days after HI, once a day	1×10^6 cells, 3 times	IP	24 h	Cells migrated mainly toward the injured hemisphere	7, 18 days	Increased mature oligodendrocytes counts. Decreased astrocytosis and microglial activation	27 days	Improved exploratory behavior, mental stress and motor function		Zhu 2014 (174)
hUCB–MSCs	P4 rats, blood injection into lateral ventricle	P6	1×10^5 cells	IVen	NA	NA	28 days	Improvements of corpus callosal thickness and myelin basic protein expression reduction. Attenuation of astrogliosis and cell death	28 days	Improved behavioral tests (negative, geotaxis test and rotarod test)	Attenuation of post-hemorrhagic hydrocephalus development by MRI. Decreased inflammatory cytokines expression in CSF (IL-1α, IL-1β, IL-6, and TNF-α)	Ahn 2013 (121)
hAECs	117 days GA fetal sheep, LPS	0, 6 and 12 h after LPS	IT 1.8×10^8 cells, or IV 9×10^7 cells, or IT 9×10^7 + IV 9×10^7 cells	7d	Cells were detected in 2 of 14 fetal brains	7 days	Decreased activated microglia in the cortex, subcortical and periventricular white matter. Decreased apoptosis in the cortex and periventricular white matter	NA				Yawno 2013 (88)

CSF, cerebral spinal fluid; GA, gestational age; HI, hypoxic–ischemia (unilateral ligatiion of the carotid artery followed by 6% oxygen systemic hypoxia); hAECs, human amnion epithelial cells; hUCB–MNCs, human umbilical cord-mononuclear cells; IP, intraperitoneal; IT, intrathecal; IV, intravenous; IVen, intraventricular; MCAO, middle cerebral artery occlusion; MSCs, mesenchymal cells; MRI, magnetic resonance imaging; NA, not applicable; P, postnatal day; UC, umbilical cord; IL, interleukin; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

and tacrolimus) improved seizures, possibly contributed by an immuno-modulatory effect of UCB–MNCs (162).

SUPPRESSION OF EXCITOTOXICITY BY UMBILICAL CORD BLOOD

Suppression of excitotoxicity is a further important subject of investigation for protecting the developing brain. The potential therapeutic effects of stem cells in animal models of excitotoxic brain injury have been examined using the *N*-methyl-D-aspartate receptor agonist, quinolinic acid, to induce apoptosis and cleaved caspase-3 and excitotoxic damage in the neocortex, hippocampus, striatum, white matter, and subventricular zone, in the newborn mouse brain. Injection of human embryonic germ cell-derived NPCs partially restores the complement of striatal neurons, with engraftment of the transplanted cells in injured sites and their differentiation into neuronal and glial cells (163). In contrast, intra-peritoneal and intravenous hUCB–MNCs administration could not promote brain repair in ibotenate-induced excitotoxic brain lesions in neonatal rats. The authors of this recent study did, however, suggest that the intra-peritoneal injection of high amounts of hUCB–MNCs may have aggravated WMI, possibly due to systemic inflammation (89).

ANTIOXIDANT EFFECTS OF UMBILICAL CORD BLOOD

There is increasing evidence that stem cells, especially young cells, possess antioxidant potential, which may then contribute to anti-apoptotic effects (164–166). A recent paper showed that hUCB–NPCs, a neuronal phenotype differentiated from collagen-adherent hUCB–MNCs, induced neuroprotection via an antioxidant effect, decreasing free radical levels by 95% (167). Human MSCs *in vitro* also scavenge oxygen and nitrogen free radicals, constitutively express antioxidant enzymes, and themselves are highly resistant to oxidative stress-induced death (166). It is still unclear whether cells derived from UCB can mediate tissue oxidative stress *in vivo*. Since oxidative stress is known to play an important role in the progression of brain injury in preterm infants, this is a crucial consideration for the ability of UCB–MNCs to mediate the progression of preterm brain injury.

VASCULAR DEVELOPMENT, INTRAVENTRICULAR HEMORRHAGE, AND UMBILICAL CORD BLOOD

Preterm infants are highly vulnerable to IVH due to their maturation-dependent vascular vulnerability, localized to the area of the periventricular germinal matrix and possibly in part due to a coagulation system deficit of prematurity (9, 168). Preterm infants predominantly develop IVH in the first week after birth (169). While grades 1 and 2 IVH cause little neurological harm, >50% of infants with severe IVH (grades 3 and 4) die or develop post-hemorrhagic hydrocephalus (PHH). The incidence of severe IVH in very preterm infants ranges from <5 to 20% (170). IVH is observed in 25% of infants with PVL and worsens WMI by increasing the amount of iron that combines with harmful free radicals and inflammatory cytokines to exacerbate injury (171, 172). The incidence of IVH has declined with current neonatal intensive care practices, but it remains an important problem, for which there is no targeted treatment (170). A recent paper demonstrated that intraventricular administration of hUCB–MSCs attenuates brain damage after severe IVH in newborn rats. The anti-inflammatory effects of MSCs (i.e., reducing the expression of inflammatory cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α) were hypothesized to contribute to the prevention of ventricular dilation and neuroprotection (121). In adult rats, IV administration of hUCB–MNCs also showed amelioration of neurologic deficits associated with intra-cerebral hemorrhage (173).

OLIGODENDROCYTES, MYELIN DEFICITS, AND UMBILICAL CORD BLOOD

Loss of pre-oligodendrocytes and hypomyelination are the principal characteristics of preterm WMI and therefore protection of oligodendrocyte lineage cells must be central to the development of any neuroprotective strategies for the preterm brain. Recently, Zhu and colleagues have shown that hUC–MSCs increase mature oligodendrocyte number and improve long-term functional outcomes following hypoxia–ischemia in postnatal day 3 rats, with engraftment of cells at lesion sites (174). It will, however be important to further elucidate whether UCB stem cells can reduce pre-oligodendrocyte injury and thereby restore myelination in fetal or neonatal animal models of WMI. There is some indirect evidence that UCB cell populations may infer benefit to oligodendrocytes and myelination. The rare but serious genetic disorders termed leukodystrophies cause degeneration of myelin and progressive neurological deterioration and, to date, the only known treatment option for leukodystrophies is early transplantation of HSCs (175). Experimentally, spontaneous myelin mutants have been used to study potential therapies. The most commonly used myelin mutant in transplant experiments is the shiverer mouse, which has a mutation in the myelin basic protein gene, and has been extensively used to study myelination by exogenous cell transplantation, including HSCs, MSCs, and oligodendrocytes progenitor cells (OPCs). OPCs can be isolated, differentiated, and expanded from both fresh and cryopreserved UCB (176, 177) offering a potential treatment option, and lay the foundations for future studies in this research field.

CLINICAL TRIALS FOR CEREBRAL PALSY

There are currently a number of clinical trials listed, or recently completed, for treatment of children with established CP. A pilot study from Hanyang University Medical Center, Republic of Korea examined 20 children aged 2–10 years with clinical CP, who were born either preterm or term, and administered peripheral autologous UCB–MNCs. Neurodevelopmental outcomes and neuroimaging studies were conducted up to 24 weeks after UCB administration, and compared with a pre-infusion baseline. Functional improvements were demonstrated in 25% of patients, and improvements in brain imaging outcomes were also noted in children with neurodevelopmental recovery. Side effects were identified in 25% of participants during infusion, treated successfully with antihistamines and hydration. Although not powered to demonstrate statistical benefit, the study showed the potential and safety of autologous UCB–MNC treatment in children (178). Between 2009 and 2012, Duke University in the USA treated 23 term newborns identified with HIE soon after birth with autologous UCB administration. The study was able to demonstrate feasibility and safety of autologous UCB re-administration in

combination with hypothermia, targeting UCB administration at 6 h. Neurodevelopmental outcomes were recorded at 1 year of age, however, a greater number of babies will be required to appropriately assess functional outcomes (179). Duke University is continuing to recruit for a larger study of autologous UCB administration for children with established spastic CP.

USE AND EFFICACY OF COMBINATION THERAPIES

A recent clinical study has assessed allogeneic UCB administered in combination with erythropoietin (EPO – itself the subject of neuroprotective trials), cyclosporine (an immunosuppressant), and rehabilitation therapy at Bundang CHA Hospital, Republic of Korea. Ninety-six children with CP aged 10 months to 10 years were treated with UCB cells, and improved cognitive and motor function were observed in all groups, including placebo, but with greater improvements in UCB + EPO children (180). Due to its design, this study does not separate the neuroprotective effects of EPO from UCB. However, combination therapy, which targets different mechanisms and therapeutic windows, may be a useful approach to treat preterm infants because of their multifactorial causes of injury and the inherent difficulties with identifying a therapeutic time frame in these infants. Indeed, ganglioside and mannitol have been shown to enhance the neuroprotective benefits of hUCB–MNCs and hUC–MSCs treatments following neonatal asphyxia in pre-clinical studies (130, 138). In contrast, despite recent promising neuroprotective outcomes of EPO in preterm cohort (181), routine clinical use of EPO, especially by high-dose, has always been hampered by its risk for retinopathy of prematurity (182). Melatonin, a powerful antioxidant shown to protect the developing brain by reducing oxidative stress following hypoxia–ischemia, with an absence of side effects, may be a candidate for co-administration with UCB (183). In term infants with HIE, hypothermia has been standard neuroprotective therapy for a number of years (184), and combination treatment of moderate hypothermia with MSCs significantly improves neuronal survival and mitochondrial activity after OGD exposure *in vitro* (185). Clinically, Cotten and colleagues recently showed that hypothermia and autologous UCB combination treatment is feasible and safe in term infants with HIE (179), and reflects that any treatment for term HIE must be considered in the context of therapeutic hypothermia. However, in very preterm and extremely preterm infants hypothermia is not currently recommended and may increase the risk of complications or death (186). A phase 1 clinical study of selected head cooling for preterm infants, born 32–35 weeks gestation, with neonatal HIE has recently been completed [NCT00620711], and the results are awaited with interest.

Other types of stem and stem-like cells may also be used for the combination therapy with UCB. UC, in addition to UCB, provides an abundant and non-invasive source of MSCs. These cells are neuroprotective in hypoxic-ischemic brain injury, and share similar *in vitro* immunosuppressive properties with bone marrow- and UCB-derived MSCs as well as mediating monocyte function to suppress T cell proliferation (130, 187). Importantly, hUC–MSCs also protect oligodendrocytes, reduce astrogliosis, and improve long-term functional outcomes in a model of preterm postnatal day 3 rats hypoxia–ischemia (174). Moreover, hUC–MSCs undergo successful cell expansion using animal serum-free culture medium, thereby removing safety concerns of animal-to-human viral transmission, further encouraging their potential for clinical application (188, 189). hAECs may present a useful therapy in combination with UCB, hypothermia, or alternate therapies. The proven anti-inflammatory properties of hAECs appear a principal mechanism to reduce preterm brain injury (88). They display both embryonic and pluripotent stem cells with abundant quantity, do not express MHC class molecules so have low immunogenicity, and do not form teratomas (190). Furthermore, the ready availability of hAECs without the need of expansion may enable them to be used for early autologous transplantation for preterm brain injury (88, 188, 189), with or without combination therapies.

OPTIMAL TIMING OF TRANSPLANTATION OF UMBILICAL CORD BLOOD

Recent experimental studies have been aimed at identifying the therapeutic window for UCB therapy. In adult rats who underwent middle cerebral artery occlusion-induced stroke, intravenous administration of hUCB–MNCs within 72 h resulted in an early functional recovery with lesion improvement, however cell administration at 120 h provided only minor functional recovery, and treatment at 14 days did not show any benefit (132). Whether a similar result can be obtained in an autologous or allogeneic setting is unknown. However, given that one of the primary benefits of UCB cells is their anti-inflammatory actions, it is likely that early intervention may be of greater benefit. Indeed, current ongoing clinical trials for neonatal HIE by National University Hospital, Singapore, and Duke University, USA are giving autologous UCB within the first 3 and 14 days, respectively after term birth asphyxia (NCT01649648 and NCT00593242). However, in preterm infants, it is difficult to know the timing of WMI that results in cystic PVL or diffuse WMI (9, 184, 191) and therefore either combination therapies, or cell preparations with multiple benefits would be most appropriate.

CLINICAL TRIALS FOR PRETERM BRAIN INJURY

No trials of neuroprotective UCB for use in treating WMI in preterm infants are currently registered in humans. A significant challenge in the design of a clinical trial for preterm infants is the question of which UCB cells to administer? As described above, it is becoming apparent that the type and quantity of specific cell types differs in preterm UCB from that in term UCB. A dose–response effect of UCB therapy for neonatal hypoxia–ischemia has been demonstrated (133, 192), but it is unclear whether a therapeutic quantity of cells can be derived from preterm UCB as the volumes obtained are low (see Umbilical Cord Blood above). Further to this, the ability to expand preterm UCB cells is not yet well described.

In preterm infants, as also discussed above, the timing of the onset, and chronic progression of WMI is usually not known. It is also not known whether to administer UCB cells before or after brain injury is identified. Administration following the identification of brain injury may provide better outcomes than administration in later childhood, due to the plasticity of the developing brain; although the evidence for this both in pre-clinical studies and clinical trials is sparse. In contrast, as cystic PVL or diffuse WMI tend to develop over days to weeks after birth (72, 169), early postnatal UCB administration "before defining

brain damage" in preterm and extremely low birth weight infants may be more efficacious. Indeed, two clinical trials administering autologous UCB to preterm infants in the first 5 or 14 days post-delivery, aiming to examine feasibility and efficacy for a variety of preterm complications, are currently underway (NCT02050971 and NCT01121328). However, in preterm infants susceptible to WMI at least until 32 weeks or more gestational age, a single administration of cells might not span an adequate period of brain protection. Thus, the need for repeated dose administration and expansion of UCB samples are further exemplified in a preterm cohort. Recently described *in vivo* cell tracking methodology using MRI, which enables the tracking of migration and distribution of magnetically labeled cells in tissues, may be useful for optimizing the time course for UCB treatment (193).

CONCLUSION

Taking into account the similarities and differences in preterm versus term brain injury, and limitations to date in stem cell studies for preterm WMI, it is apparent that a number of considerations apply before UCB treatment could be extended to infants born preterm. The clear advantage of undertaking UCB administration in a preterm cohort is the relative plasticity of the developing brain in immature infants, and potential for regeneration. However, there are current disadvantages that must be overcome. Studies to date suggest that early cell administration post-injury achieves favorable therapeutic outcomes, but a current lack of sensitive diagnostic tools and inability to accurately determine the onset of preterm brain injury remains problematic. Work to define the most appropriate time for therapeutic intervention is needed. Additionally, the specific cells present in UCB responsible for brain protection are not yet characterized and the complications of pregnancy that are often co-morbidities with preterm birth, such as uteroplacental inflammation or IUGR, may alter the cellular composition of UCB. A handful of published work suggests that preterm UCB cell number, cell total population and cell maturity is different to that in term UCB, which may not provide the expected benefit that has been observed using term hUCB in experimental animal and clinical studies. It is therefore currently not known whether autologous or allogeneic UCB cell administration would confer optimal benefit in a preterm cohort, or whether expansion of specific cell types should be considered and pursued. Thus it remains that UCB holds strong promise for the treatment of preterm brain injury in the neonate, but fundamental questions must be answered with appropriately designed experimental animal and clinical studies prior to large-scale randomized clinical trials for preterm brain injury.

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Activity-based therapies for repair of the corticospinal system injured during development

Kathleen M. Friel[1,2]*, Preston T. J. A. Williams[3], Najet Serradj[3], Samit Chakrabarty[4] and John H. Martin[3,5]*

[1] Department of Neurology, Brain and Mind Research Institute, Weill Cornell Medical College, New York, NY, USA
[2] Burke Medical Research Institute, White Plains, NY, USA
[3] Department of Physiology, Pharmacology and Neuroscience, City College of the City University of New York, New York, NY, USA
[4] School of Biomedical Sciences, Faculty of Biology, University of Leeds, Leeds, UK
[5] The Graduate Center of the City University of New York, New York, NY, USA

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Claire Gibson, University of Leicester, UK
Ann-Christin Eliasson, Karolinska Institutet, Sweden

***Correspondence:**
Kathleen M. Friel, Burke Medical Research Institution, White Plains, NY 10605, USA
e-mail: kaf3001@med.cornell.edu;
John H. Martin, Department of Physiology, Pharmacology and Neuroscience, City College of the City University of New York, 160 Convent Avenue, New York, NY 10031, USA
e-mail: jmartin@ccny.cuny.edu

This review presents the mechanistic underpinnings of corticospinal tract (CST) development, derived from animal models, and applies what has been learned to inform neural activity-based strategies for CST repair. We first discuss that, in normal development, early bilateral CST projections are later refined into a dense crossed CST projection, with maintenance of sparse ipsilateral projections. Using a novel mouse genetic model, we show that promoting the ipsilateral CST projection produces mirror movements, common in hemiplegic cerebral palsy (CP), suggesting that ipsilateral CST projections become maladaptive when they become abnormally dense and strong. We next discuss how animal studies support a developmental "competition rule" whereby more active/used connections are more competitive and overtake less active/used connections. Based on this rule, after unilateral injury the damaged CST is less able to compete for spinal synaptic connections than the uninjured CST. This can lead to a progressive loss of the injured hemisphere's contralateral projection and a reactive gain of the undamaged hemisphere's ipsilateral CST. Knowledge of the pathophysiology of the developing CST after injury informs interventional strategies. In an animal model of hemiplegic CP, promoting injured system activity or decreasing the uninjured system's activity immediately after the period of a developmental injury both increase the synaptic competitiveness of the damaged system, contributing to significant CST repair and motor recovery. However, delayed intervention, despite significant CST repair, fails to restore skilled movements, stressing the need to consider repair strategies for other neural systems, including the rubrospinal and spinal interneuronal systems. Our interventional approaches harness neural activity-dependent processes and are highly effective in restoring function. These approaches are minimally invasive and are poised for translation to the human.

Keywords: corticospinal tract, activity-dependent development, motor cortex, motor cortex stimulation, cerebral palsy, mirror movements

INTRODUCTION

The corticospinal (CS) system is the principal motor system in humans and many mammals for skilled movements. Damage to this system results in significant motor impairments. In maturity, loss of function after CS system injury, such as weakness and paresis, predominates. Essential coordination necessary for even the simplest of skilled movements, like reaching, grasping, and feeding, is also typically lost. Loss of motor skill and coordination are thought to be due primarily to the loss of the direct projections of the corticospinal tract (CST) from motor cortex to spinal cord motor circuits. CS system damage during development also leads to the gain of aberrant and debilitating functions that are key motor impairments in cerebral palsy (CP) (1). These include hyperreflexia and spasticity as well as aberrant limb and postural coordination. Mirror movements are also common in CP, particularly hemiplegic CP (2). Gain of aberrant functions in CP likely reflects several inter-related processes, including a loss of flexible and individuated muscle control replaced by relatively fixed motor synergies, and hyperreflexia and spasticity (3–6). Our research has identified another important factor contributing to impaired control – development of misprojections between spared cortical motor pathways and spinal and brain stem motor centers. This is maladaptive developmental miswiring of CS motor circuits, both of the injured system and the system that is spared. A critical question that we will focus on in this review is why the loss of CS connections due to perinatal brain trauma leads to maladaptive development of the surviving connections. Whereas it has been well-established that the brain is extraordinarily plastic early in development, beyond that there is a paucity of mechanisms to inform why miswiring occurs and how best to intervene. Our work in animals provides an understanding of the mechanisms underlying miswiring and a strategy to repair abnormal CS connectivity.

In this review, we will first examine normal CS system development, including new findings on genetic developmental cues governing the laterality (i.e., contralateral or bilateral) of CST projections and motor function. Knowledge of the basic underlying genetic mechanisms of CST development can be leveraged to help inform why brain injury in CP can have a profound effect on wiring of the CST and the gain of aberrant functions. Next, we consider the role of neural activity-dependent processes in refinement of the CST from a bilateral to predominantly contralateral motor pathway. In the context of normal development, we briefly consider co-development of the CST and the rubrospinal system, the other major system for limb movement control. Limb movement normally reflects the dual actions of the cortical and rubrospinal systems (7, 8). Although we do not as yet know the specific reaction of the rubrospinal system to damage of the CS system, during the period when CS system damage leads to CP is when the rubrospinal system seems to play a unique role in limb control. Then, we will show how knowledge of normal development informs the question of why particular CST misprojections occur after early brain injury. Finally, we discuss how harnessing activity-dependent synaptic competition, which is key to normal development, leads to restoration of CST connections and recovery of motor skills in an animal model of hemiplegic CP. The aim of this review is to present the mechanistic underpinnings of motor system development derived from animal models, not to summarize therapeutic options for patients with CP. Nonetheless, toward the end of this review, we discuss strategies for neural repair based on our animal model that can be readily implemented in humans because they can be non-invasive or minimally invasive.

GENETIC FACTORS HELP ESTABLISH THE LATERALITY OF CST SPINAL PROJECTIONS DURING DEVELOPMENT

To understand why the CST develops misprojections in CP and how this relates to the motor impairments, we must examine first the mechanisms of normal development. As for other neural systems, the CST depends on the interplay between genetics, neural activity, and experience to achieve appropriate circuit formation and performance. Genetic mechanisms specify which cortical neurons develop to become CST neurons, and others develop to become interneurons and other projection neurons (9–11). Genetics also plays a key role in guiding CST axons to their targets in the spinal cord. Here, we focus on axon guidance because this is both important for normal wiring of connections and sets the stage for understanding why CST projections go awry after perinatal injury.

Diverse guidance molecules shepherd growing CST axons to their brain stem and spinal targets, including decussation of most CST axons from the medullary pyramid to the contralateral spinal cord white matter (12). CST neurons have complex sets of receptors that enable the guidance molecules present throughout the developing central nervous system to act as ligands to affect steering of the growth cone to ensure that CST axons reach their intended targets (13). The majority of CST axons reach the contralateral spinal segments after decussating in the pyramid and then project extensively into the gray matter on the same side as the descending spinal axons. This is the predominantly contralateral projection, characteristic of normal CST development.

In the spinal cord, the receptor tyrosine kinase EphA4, along with its ligand EphrinB3, restricts CST outgrowth from the contralateral to the ipsilateral spinal gray matter and thereby helps ensure a predominantly contralateral termination pattern (14–17). EphA4 receptors on the CST growth cone bind to EphrinB3 on midline glial cells, which leads to axonal retraction. Evidence suggests that the overall level of EphA4 is used during development to regulate the amount of recrossing of the CST in the spinal cord and, in turn, the extent to which the developing CST establishes bilateral spinal terminations (17). In genetically normal animals – and likely in humans, as well – this mechanism results in a significant percentage of CST axons that project into the gray matter and recross within the spinal cord early in development. In the cat, which has been studied extensively, early-developing ipsilateral CST projections, which may be as much as 50% of the contralateral projection (18), are subsequently refined to a smaller number that is maintained into maturity (**Figure 1**). This refinement (discussed further below) establishes the predominantly contralateral CST projection pattern. Similarly, in normal human development, single-pulse transcranial magnetic stimulation (TMS) of motor cortex in neonates evokes bilateral responses, suggestive of strongly bilateral projections, whereas by 6 months of age, only contralateral responses are evoked (19, 20). The amount of recrossing also seems to vary across different animal species, with the monkey showing more than the cat or rodent (21, 22); although this has not been studied systematically.

Mouse genetics can be leveraged to study the question of development and maintenance of CST laterality, and importantly, inform the functional significance of bilateral CST projections for movement control. When the gene for EphA4 is eliminated selectively in the forebrain of mice, but not in the brain stem and spinal cord, the CST develops a strongly bilateral projection to the spinal cord (**Figure 2A**). These bilateral CST axons terminate on normally organized spinal circuits within the spinal cord (16, 17). An aberrant bilateral CST projection in mature mutant mice underlies robust bilateral CS motor circuit changes and bilateral voluntary behaviors. With bilateral CSTs, electrical stimulation of the motor cortex evokes bilateral limb muscle responses (16, 17), and a bilateral motor representation in motor cortex (17). In the knockout mouse, we characterized the motor map in terms of the number of sites where stimulation at threshold evoked mirror movements (identical movements of each forelimb). The wild type mice have no sites with this property, while approximately 80% of sites in all knockout mice evoked mirror movements (**Figure 2B**) (17). This shows that the aberrant bilateral CST projections in maturity are effective in activating spinal motor circuits bilaterally.

Does development of a bilateral CST and a mirror movement representation in motor cortex produce clear and consistent bilateral voluntary motor responses? This question was addressed by examining two motor behaviors that WT mice produce using unilateral limb movement: obstructed locomotion, where the animal steps over obstacles, and exploratory reaching (**Figure 3**). Both behaviors are voluntary. In obstructive locomotion, the animal uses obstacle sensory information (e.g., obstacle height and distance) to modify the gait pattern to clear the obstacle (23). And in exploratory reaching, the animal stands on its hind legs and reaches to explore the walls of the enclosure within which it is located (17).

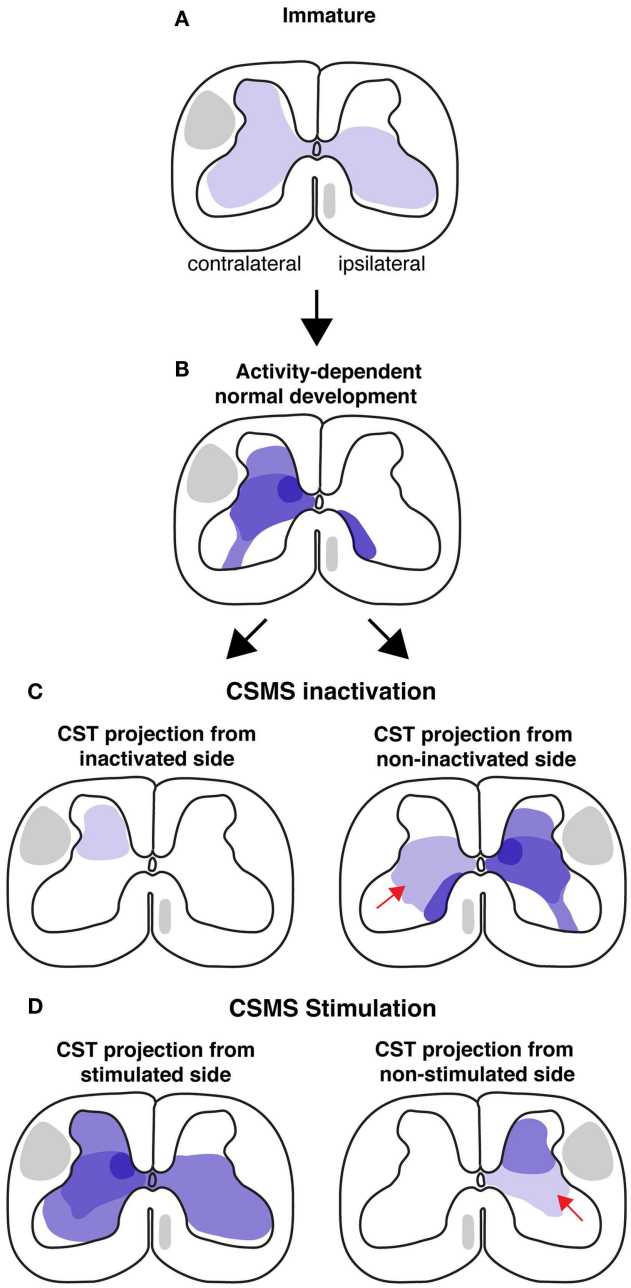

FIGURE 1 | Schematic illustrations of CST development in cat model during normal conditions and after activity manipulations. Each illustration is a semi-schematic representation of the CST cervical projection pattern. **(A)** Immature projection. Contralateral and ipsilateral are with respect to the motor cortex of origin of the pathway. The immature pattern is bilateral. **(B)** Normal development results in a predominantly contralateral projection, with multiple sites of high projection density (e.g., intermediate zone). **(C)** CSMS unilateral inactivation results in a greatly contracted termination space (left). The non-inactivated side develops aberrant ipsilateral misprojections. **(D)** Electrical stimulation of the corticospinal motor system (CSMS) results in maintenance of many of the extensive terminations observed in early development, as shown for the stimulated side (left). The non-stimulated side (right) develops fewer ventral projections (arrow).

WT animals use alternate stepping over the obstacle and reach with one or the other forelimb (**Figure 3**). By contrast, EphA4 knockout mice rarely use alternate stepping to clear the obstacle, but instead use bilateral synchronous forelimb and hind leg movements, resembling hopping. During exploratory reaching, EphA4 knockout most commonly use both arms simultaneously, resembling mirror movements in people with CP (2). To summarize, by manipulating EphA4 signaling genetically we show the behavioral significance of ipsilateral CST misprojections (17). As we discuss below, ipsilateral CST projections become highly reactive after unilateral brain injury in CP. Similar to the EphA4 knockout mice, with a maladaptive expansion of the ipsilateral CST, many people with CP express mirror movements. Our findings using a genetic model further show that bilateral CST projections, not aberrant bilateral spinal circuits, can explain the presence of mirror movements.

THE ACTIVITY OF THE MOTOR SYSTEMS AND LIMB USE REFINE CST PROJECTION PATTERNS

Guidance molecules help to establish a coarse early spinal termination pattern of the CST. This pattern is subsequently refined later in development into the mature pattern (**Figure 1**). What is the mechanism by which the early coarse pattern of connections is refined? Since refinement occurs postnatally as the animal begins to express skilled motor behavior, we focused on the role of activity-dependent processes in establishment of the proper patterns of CST connections with spinal circuits. Our studies in the cat demonstrate an important role for the constitutive level of activity of the CS system in each hemisphere in establishment of spinal connections and a role for limb use, which likely reflects activity patterns. When activity in one motor cortex is blocked pharmacologically by infusing the $GABA_A$ agonist Muscimol during an early sensitive period, CST axons withdraw their projections [**Figure 1C**; left spinal cord; (24)]. Preventing use of one limb during a similar period has a similar effect on development of contralateral CST projections (25). By contrast, when electrically stimulated, CST axons extend more projections (**Figure 1D**; left spinal cord)(26). This shows the importance of activity-dependent factors in shaping the pattern of CST spinal projections and further suggests interactions – possibly competitive (see below) – between the CS systems from each hemisphere.

The unilateral activity interventions have major bilateral effects. The non-inactivated side (**Figure 1C**, right spinal cord) develops a normal contralateral projection but, additionally, an aberrant ipsilateral projection (**Figure 1C**, right, red arrow). Importantly, a bilateral CST from the less-affected side in often considered pathognomonic for hemiplegic CP (discussed below). Similarly, the non-stimulated side develops a diminished projection, with fewer intermediate and ventral projections (**Figure 1D**; bottom row, red arrow). These findings, together with more limited results from bilateral treatment [activity blockade; (27)], indicates the importance of the relative amount of activity in the developing CS system. We will see below that this is a key finding for understanding the pathophysiological mechanisms underlying CP.

Thus, guidance mechanisms driven by genetic regulation of EphA4 initially help establish the density of ipsilateral CST axons.

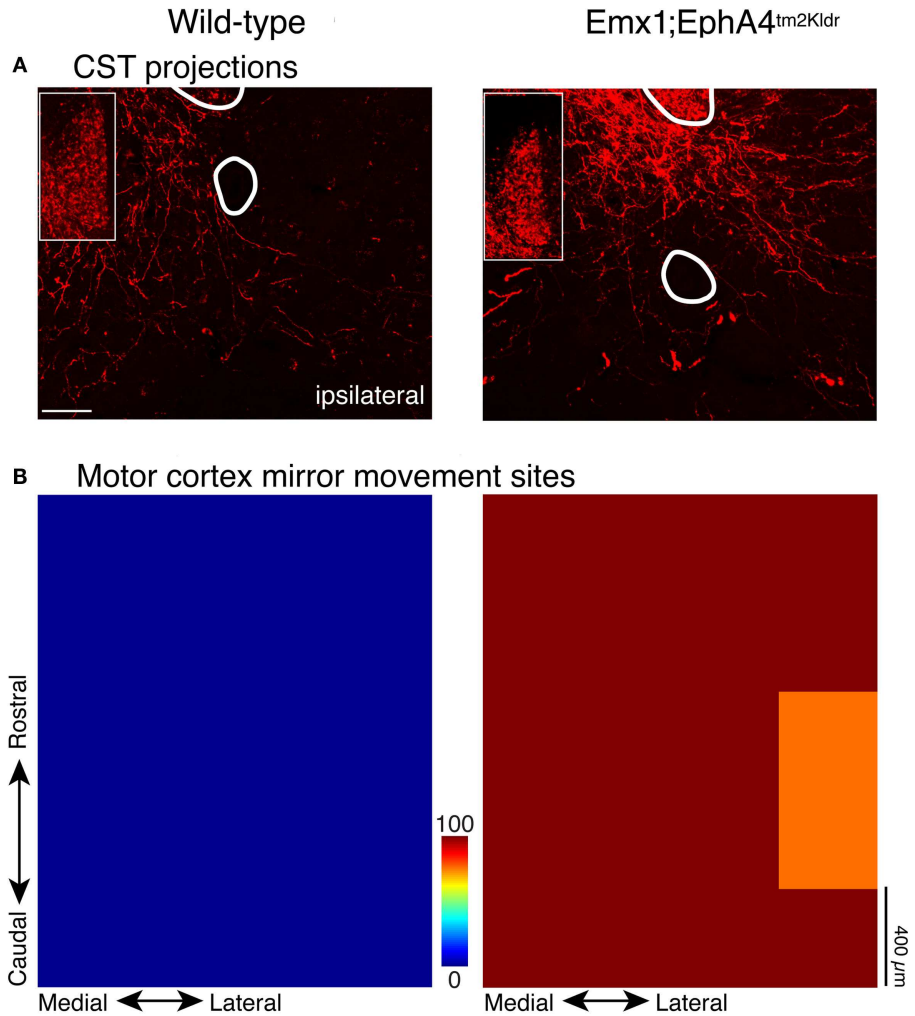

FIGURE 2 | CST in wild type and EphA4 conditional knockout mouse. Modified from Serradj et al. (17). **(A)** CST terminations within the intermediate zone flanking the midline. Wild type mice (left) have a predominantly contralateral projection, whereas the mouse with conditional elimination of the EphA4 gene in the forebrain (right) has a strongly bilateral projection. The insets show labeling in the dorsal column, which is the location of the CST in the mouse; labeling is comparable in the two mice. **(B)** Color-coded representations of sites in motor cortex where electrical stimulation evoked bilateral movements (e.g., mirror movements; average of five mice in each category). Whereas no "mirror sites" existed in the wild type mice (left), they were ubiquitous in the mutant mice (right).

Activity- and use-dependent processes subsequently shape the pattern initially established by genetic mechanisms. A plausible developmental strategy is that the more liberal the recrossing of CST axons, the greater opportunity for bilateral connections to be supported by early-developing bimanual limb movements. For example, if the developing child does not engage in extensive bimanual control, then more ipsilateral CST projections are eliminated compared with a child that uses more bimanual control. Further, the animal data provide support for a developmental rule whereby the more active (and more extensively used) connections of the developing CS system overtake less active/used connections. This helps explain the normal development of a predominantly crossed CST and the elimination of the early ipsilateral projections. As we discuss below, this activity-based rule also helps explain misprojections in CP.

MOTOR REPRESENTATION DEVELOPMENT IN MOTOR CORTEX AND THE ROLE OF THE RED NUCLEUS IN EARLY MOVEMENT CONTROL

The CST is one of many motor systems of the brain and spinal cord (28). In maturity, the CST functions together with the red nucleus (RN), which gives rise to the other major descending pathway for limb control, the rubrospinal tract (7, 8). The CS system is thought to play a greater role in more flexible and adaptive movements, and the rubrospinal system, in more automatic limb movements. Whereas the rubrospinal system has been speculated to play a role in recovery after CST damage in maturity (29, 30), its role in motor development in health and disease is not yet known (4). Interestingly, the rubrospinal system may have even more anatomical and functional prominence early in human development than it does later in life (31). We have

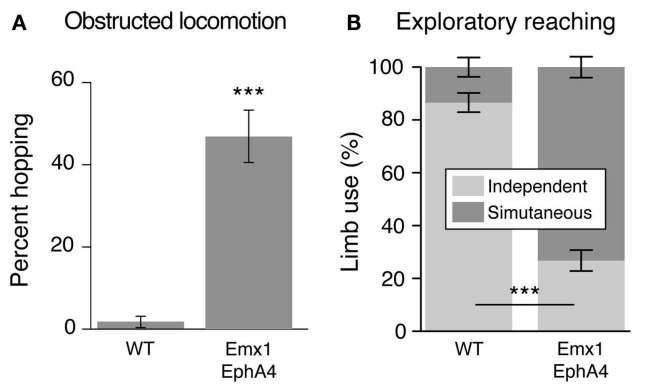

FIGURE 3 | The conditional EphA4 knockout mouse displays bilateral voluntary fore limb movements. Modified from Serradj et al. (17). **(A)** Obstructed locomotion. Wild type mice show alternate stepping whereas the EphA4 conditional mutant mice show synchronous fore limb movements (hopping) during obstructed locomotion. **(B)** Exploratory reaching. Wild type mice show independent limb use whereas the EphA4 conditional mutant mice show synchronous limb, or mirror movements, during reaching.

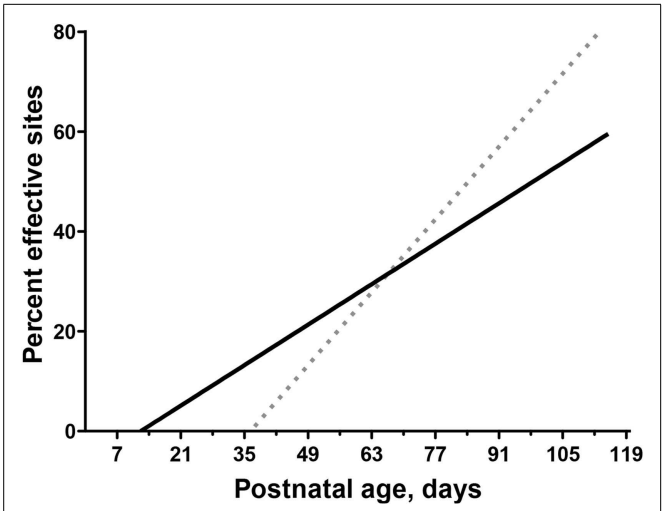

FIGURE 4 | Differential motor map development in motor cortex and red nucleus. Modified from Williams et al. (32) and Chakrabarty and Martin (35). Comparison of age-dependent increases in the percentage of sites in motor cortex (dotted line) and RN (solid line) where stimulation produced a contralateral motor response (termed effective sites). The linear fit is shown for both motor cortex ($Y = 1.54x - 68.62$) and RN ($Y = 0.66x - 10.03$).

investigated development of the rubrospinal system in relation to CST development.

When the developing CS and rubrospinal systems are directly compared in an animal model, it has been shown that the rubrospinal system matures earlier than the CST (32). Early behavioral contributions of the CS and rubrospinal system can be evaluated by comparing development of their motor representations. The motor cortex motor map is a good indicator of CS system function (33). In the cat, for example, the motor cortex map comes "online" at about postnatal week 7 (**Figure 4**, dotted regression line) (34, 35) and this is when several measures of voluntary movement control – including object manipulation, and social/play interactions – develop (36, 37). Map development plateaus at about postnatal week 12, and this is also when expression of voluntary control stabilizes. Surprisingly, development of the red nucleus motor map begins earlier than the motor cortex motor map (**Figure 4**). In motor cortex, proximal limb joints are represented at younger ages then distal joints, also paralleling late development of distal skills (35). By contrast, in the red nucleus distal as well as proximal forelimb muscles are represented at the outset. Taken together, these findings suggest that the rubrospinal tract is important for establishing the rudiments of motor skills before the CST has come online.

An important question, yet to be resolved, is the extent to which these two motor systems interact during development. Emerging evidence from our laboratory suggests that there is an activity-dependent interaction between the developing corticorubral and rubrospinal projections (38, 39). When motor cortex activity is blocked during early development, the red nucleus on the side of the inactivation may have an enhanced development, while development of the red nucleus motor map on the opposite side is remarkably impaired. This finding hints at a competition between the developing rubrospinal and CS systems. Red nucleus compensation on the injured side could be part of the biological basis of partial recovery after developmental cortical injury. That the rubrospinal system precedes CS system development suggests that it may not be as prone to the same miswiring as the CST following early activity manipulations because its sensitive period is earlier.

MECHANISMS OF MALADAPTIVE DEVELOPMENT OF THE CORTICOSPINAL SYSTEM AFTER UNILATERAL INJURY

As expected, cortical and white matter injury destroys the cells of origin and connections of the CS system. In doing so, injury will not only disrupt CST function but system-wide development as well. With a predominantly unilateral injury, what is not well understood is why damage on one side leads to development of misprojections of the spared CST from the uninjured hemisphere. Our studies strongly suggest that the same activity-dependent mechanisms that ensure optimal CST development under normal conditions can become maladaptive after perinatal injury to the CS system.

With an initial bilateral organization, the typical-developing CST eliminates most ipsilateral spinal projections (**Figure 1**). As discussed above, the initial density of ipsilateral CST projections is regulated, in part, by a genetic mechanism (EphA4) and the subsequent reduction in ipsilateral projections reflects an activity-dependent refinement process (12). **Figure 5A** shows the normal contralateral (top) and ipsilateral (bottom) projections. When the activity of the CS system in one hemisphere is reduced, by intracortical infusion of muscimol, the affected hemisphere is not able to establish its normal projection pattern contralaterally (**Figure 5B**, top). In a reciprocal manner, the ipsilateral projections of the CS system in the other hemisphere are better able to compete with the less active CST for synaptic connections with spinal cord neurons (**Figure 5B**, bottom). In these experiments, CST axons from

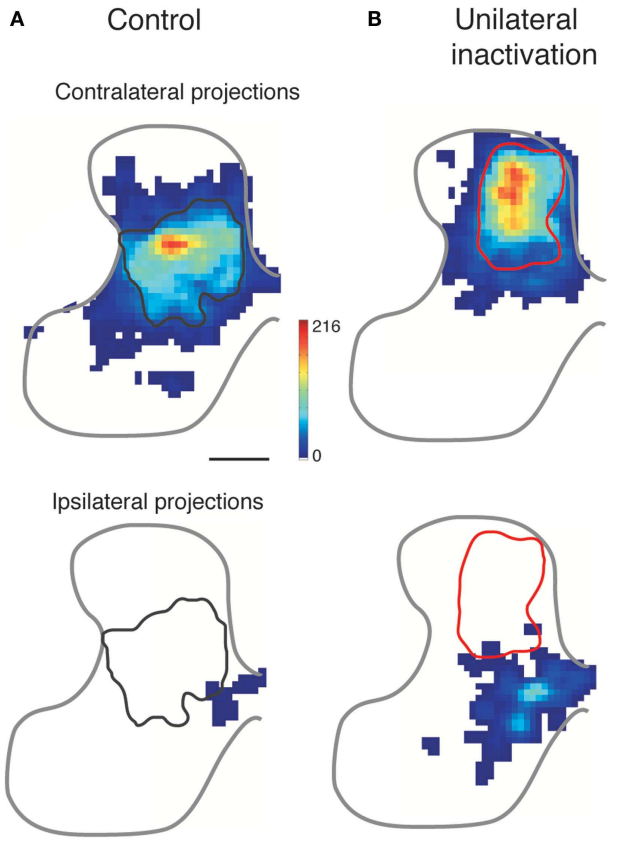

FIGURE 5 | Effect of activity blockade on CST development. Modified from Friel and Martin (24). Color-coded representations show local CST density in the cervical gray matter. The normal contralateral CST projection **(A)** is densest to the intermediate gray matter, and does not overlap with the ipsilateral projection from the other hemisphere, which is located medially. After perinatal inactivation **(B)**, the contralateral projection shifts dorsally and the ipsilateral projection from the other hemisphere expands laterally. The gray and red outlines mark the densest territory of contralateral CST projections for normal development (left column) and after unilateral CST inactivation (right column), respectively. The contralateral and ipsilateral "heat maps" are plotted with the same color scales.

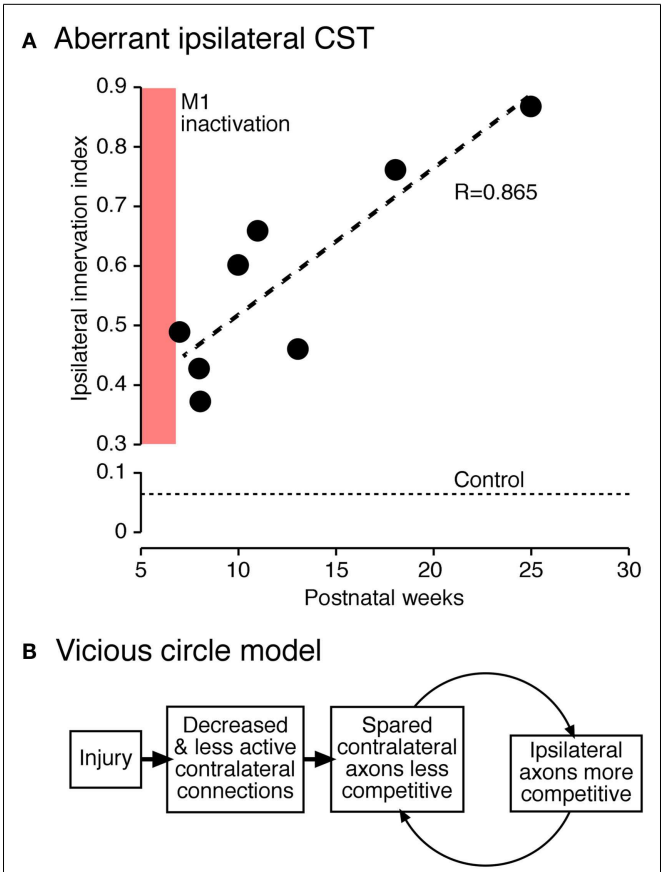

FIGURE 6 | Vicious circle hypothesis (42). **(A)** Aberrant ipsilateral corticospinal terminations further develop postnatally. The graph plots the ratio of local density of ipsilateral and contralateral CST terminations. **(B)** Hypothesis of progressive decline of less active connections and complementary augmentation of more active connections.

only a small portion of the forelimb area of motor cortex were labeled. Nevertheless, comparison of the control and inactivated animal revealed a 3.6-fold increase in the spatial extent of the ipsilateral projections with a peak approximately 1/3 that of the contralateral projection. Thus, the active CS system maintains, and likely strengthens, its early bilateral organization (**Figure 5B**, bottom). The incursion of abundant CST outgrowth into the ipsilateral spinal gray matter greatly impedes development of the less active CST. The contralateral axons of the less active CST are prevented from typical outgrowth and synapse formation (24, 40). Further, there is a dorsal shift for the inactivated CST (24, 40, 41). We do not yet know the mechanism of this circuit change. However, it is apt to be functionally significant because this dorsal area of the dorsal horn is more concerned with the processing of somatic sensory information than motor output (28). Because of this location, the impaired CST may have reduced access to spinal motor circuits and, as a consequence, is much less functional. The question remains in the animal model if this aberrant dorsal CST projection is maladaptive. By not targeting their proper spinal circuits, this projection could underlie aberrant contralateral control, such as spasticity, incoordination, and reflex radiation.

As development progresses, the ipsilateral CST from the active side continues to establish connections at the expense of the contralateral projection from the inactive side. We showed that this process progresses even after activity is restored to the previously silenced side (**Figure 6**). We propose that perinatal injury before elimination of early ipsilateral CST projections creates a "vicious circle" of further loss of injured and gain of spared CST fibers (42). This is due to impaired capacity of the injured side to maintain connections, and a concomitant robust reactive increase in ipsilateral CST projections from the undamaged hemisphere. The underlying mechanism may be activity-dependent synaptic competition, with a loss of competitive ability of the injured side and a gain of competitive ability by the undamaged (or less damaged) side.

We equate the less active CST in our model with the damaged CST after a lesion. The affected limb is used less than normal,

thus the surviving fibers of the CST serving that limb would be expected to be less active. There also would be reduced afferent feedback because of the paucity of movements, and possibly reorganization of proprioceptive inputs to the spinal cord (43, 44). Conversely, the unaffected (or less affected) limb is used more and the undamaged CST would be expected to be more active. Thus, experimentally manipulated activity changes both inform mechanism (e.g., activity may be more important than the physical loss of connections due to damage) and are also a reasonable model for what happens after a unilateral lesion. Damage to one hemisphere, and the associated lack of limb use, can thus lead to a dual vulnerability. The damaged side loses much of its contralateral connections to injury, while the undamaged (or less damaged) side "fills in" these denervated territories with ipsilateral misprojections and further constrains development of the remaining contralateral CST projections.

We hypothesize that the aberrant, and possibly maladaptive, ipsilateral CST in people with hemiplegic CP reflects a basic mechanism for synaptic stabilization that goes awry after injury. With injury, more robust than loss of activity as in our animal model, the loss of connections and lack of affected limb use makes this side less able to compete for synaptic connections with spinal motor circuits than the uninjured system. By contrast, the less-affected CST concomitantly gains synaptic competitive ability. When damage occurs after the early ipsilateral CST projections are eliminated, reduced synaptic competition may continue to play an important role in the evolving motor impairment. In maturity, loss of connections of the damaged system is counterbalanced by a small reactive increase in spared ipsilateral CST projections from the undamaged hemisphere (45, 46) and a reactive increase in proprioceptive afferent projections (47, 48). At this point after an injury in maturity, denervated spinal circuits would be driven more by afferent fibers and ipsilateral CST projections. This too likely results in motor impairment.

INTERVENTIONAL STRATEGIES

Knowledge of the pathophysiology of the developing CST after injury informs interventional strategies for protecting the damaged system from further loss of connections and function during development. This knowledge is also helpful in devising strategies for repairing aberrant CS circuits once development has taken place. As discussed, a well-known misprojection of the CST in patients with hemiplegic CP is development of ipsilateral misprojections. Eyre et al. (49) have shown that perinatal injury results in the progressive loss of the damaged CST and maintenance and possible strengthening of the ipsilateral CST; this is akin to the vicious circle discussed above (42). The net outcome is that the spared side develops an aberrant bilateral CST. We infer from our genetic model [**Figures 2** and **3**; (17)] that one consequence of these bilateral misprojections in CP is mirror movements (2). Other misprojections are likely to play a role in impaired intralimb coordination, reflex radiations, and spasticity. We hypothesize that during the period when the ipsilateral projections are strengthening after perinatal injury, activity-dependent competition is "out of control" driving down contralateral function of the affected side and establishing stronger ipsilateral projections from the non-affected side.

Using the cat model described in the previous section, we aimed to repair the aberrant pattern of connections and restore function by harnessing activity-dependent processes. According to our competition hypothesis, either improving the capacity of the injured system to compete for spinal synaptic connections or diminishing the unaffected system to compete, should help restore a more normal CST pattern of connections and improve function. We tackled both approaches and achieved differential success that can be explained on the basis of a dosing effect.

In two separate studies, we showed that promoting the activity of the injured system (26) or decreasing the activity of the undamaged system (24) immediately after the period of injury leads to partial repair of the CST and restoration of skilled motor function. These remarkably effective activity-based interventions provide proof-of-principle of the capacity to harness activity after developmental impairment to repair CST connections and function. We next took a more clinically minded approach and used constraint of the less-impaired limb at two time points, with and without behavioral training, to determine if behavioral approaches are similarly effective. We examined three conditions: (1) constraint immediately after the impairment; (2) constraint immediately after the impairment plus intensive motor training (reach to grasp); and (3) delayed constraint with intensive motor training. It is important to recognize that the effects of early developmental impairment [e.g., **Figure 6B**; CST abnormalities and motor control impairments; (24, 50)] are permanent without further intervention. Remarkably, whereas all three interventions result in comparable repair of the contralateral CST (**Figure 7A**), there were differences in the motor cortex motor maps and spinal interneuronal function that accounted for whether or not animals showed behavioral recovery.

With constraint and intensive training both initiated early in development (**Figure 7**), there was restoration of the motor cortex motor map; a complete representation of contralateral forelimb joints was present (**Figure 7B**) (41). Thus, representational plasticity, which occurs throughout the animals lifetime (35, 51), helps to provide an effective substrate for contralateral limb control. Although the absolute numbers of spinal cholinergic interneurons were not different, side-to-side, we also observed relative increases in spinal cholinergic interneurons (**Figure 7C**; boxed region compares interneurons in the intermediate zone). These interneurons may play a key role in relaying CST signals to motoneurons (52–55). Together, the motor map and interneuronal changes support behavioral recovery. Without intensive training (**Figure 7**, middle column), the motor cortex motor map failed to show representational plasticity; the motor map was essentially lost (41). Further, interneuronal numbers did not improve. Together, the absence of the map and insufficient interneuronal changes could have prevented behavioral recovery. Clearly, restraint alone is not therapeutic; it needs to be combined with training.

Importantly, delayed intervention into the feline equivalent of young adolescence led to restoring the motor map but not cholinergic spinal interneurons [**Figure 7**, third column; (41)]. Since this animal group did not recover function, but did show repaired CST projections and M1 motor map, it shows the importance of spinal circuitry and possibly other pathways (e.g., rubrospinal tract) in normal and recovered function. In the cat, spinal cholinergic

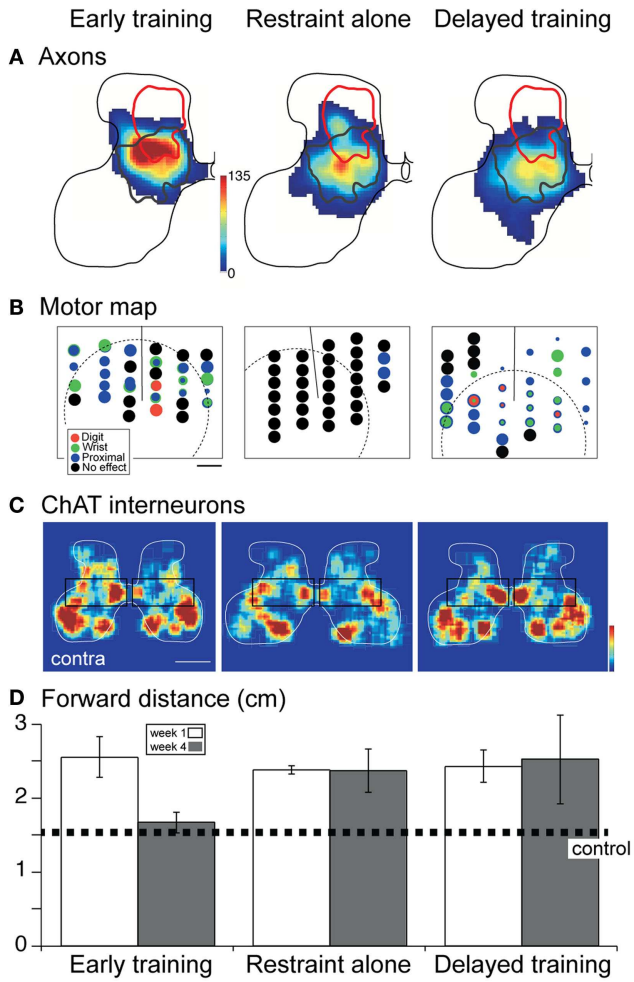

FIGURE 7 | Effect of constraint of the unimpaired limb on CST projections, the motor cortex motor map, spinal cholinergic interneurons, and skilled behavior. Modified from Friel et al. (41). **(A)** Distribution of CST projections in the cervical enlargement. Density of projections is represented by a color scale, with red corresponding to the densest projections. All "heat maps" are plotted with the same color scale. The red line corresponds to the aberrant dorsal projection of the inactivated CST (as shown in **Figure 6**), whereas the dark gray line is the normal distribution (**Figure 6**). **(B)** Motor cortex motor map. Each site examined for a motor response with stimulation is marked by a circle. The threshold for evoking a contralateral motor response is represented inversely by circle diameter. Black corresponds to no response sites. **(C)** Color-coded representations of local cholinergic interneuron density. Boxes highlight differences in relative density. Contralateral compared with ipsilateral density is greatest for early training. **(D)** Forward distance is a measure of reaching accuracy. The dotted line marks normal performance. Over-reaching is characteristic at 1 week for all groups. Only the early trained group shows recovery to normal distances; performance of the other groups remains errant.

interneurons develop during the same period when the CST connections are being refined (53). We hypothesize that there is a critical period for spinal cholinergic interneuron development, under CST activity control (41). The capacity to restore this interneuronal function is restricted to the early intervention period. This demonstrates the importance of repair strategies that take other neural systems into account. The rubrospinal system is another potentially important system that should be considered in repair after perinatal damage.

Several studies have demonstrated efficacy of activity-based therapies for children with hemiplegic CP (56–61). These therapies – either constraining the less-affected upper limb and intensively training the affected upper limb, or intensively training bimanual upper limb movements – improve hand function in children with hemiplegia. Since intensive therapy requires children to engage and follow instructions, these therapies are typically delivered to school-aged children. It is hypothesized that, just as in our cat model, these therapies increase activity of the impaired side, thus increasing the competitive strength of the impaired side against the less-impaired side. Although these activity-based therapies improve upper limb control, function is not fully restored. Hand function is still most often substantially impaired compared with typically developing children. Our studies in the cat model indicate that early intervention, before the less-impaired CST secures a strong competitive advantage over the impaired CST, may be necessary for mitigating the motor dysfunction of CP.

Although clinical application of activity-based therapies is challenging in young children, there have been efforts to translate therapy to this population. Gordon et al. (62) have developed a caregiver-driven bimanual therapy for toddlers that significantly improved bimanual hand use and performance of functional tasks. Several studies have indicated that enriched environments that encourage infants to move and reach can improve motor outcomes in very young children who have, or are at risk of developing, CP (63–65).

Non-invasive brain stimulation may be a viable strategy for balancing activity-dependent competition between the two sides of the brain after unilateral brain injury. Repetitive transcranial magnetic stimulation (rTMS) has been shown to boost the amount of motor improvements seen after gait or hand training (66, 67). Recently, transcranial direct current stimulation (tDCS) has emerged as a preferred method of non-invasive brain stimulation due to its excellent safety profile, portability, and low cost. tDCS has been tested in a small number of children with hemiplegia, and has been shown to be safe and tolerable (66). Non-invasive brain stimulation may emerge as a potential therapy for very young children, to balance interhemispheric competition before the damaged side loses its foothold in the developing motor system. However, safety is an important concern that must be evaluated further. The use of brain modeling is likely to be an important step in determining the safety of brain stimulation in young children (66, 68).

PERSPECTIVES AND CONCLUSION

We envisage two distinct periods of intervention, where repair of the damaged CS system can occur. Very early during development, during the so-called critical period, activity-based approaches can have a neuroprotective effect by enhancing the ability of the damaged CST to establish spinal connections. This is likely the most effective period since it is directed to facilitate spinal circuit development. Extrapolating from the cat data, in the human this

would correspond to the first 6–12 postnatal months (19). Later in development, after aberrant CST connections are established and become permanent if not treated further, there is still a robust capacity to repair. CST growth to original targets and partial elimination of aberrant ipsilateral misprojections can occur. Indeed, intact CST axons are capable of sprouting into the denervated side of the spinal cord in maturity (45, 46, 69). However, the efficacy is strongly reduced after the critical period and is insufficient to restore significant function unless promoted. There also is robust representational plasticity of the motor cortex (41). Importantly, in our model, behavioral therapies do not appear to be effective in repairing aberrant spinal interneuronal circuits later in development. It is plausible that direct activity manipulations, such as motor cortex stimulation (45, 46), are needed to coax spinal interneurons to improve function.

A critical question is if the ipsilateral CST after unilateral development lesion can be adaptive? By themselves, ipsilateral CST projections target different sets of spinal cord laminae compared with their contralateral counterparts (45, 69). As a consequence, different somatic sensory and motor circuits will be engaged by the ipsilateral and contralateral tracts. This points to limitations in function but not necessarily maladaptive functions. Further, the ipsilateral projection in concert with the contralateral projection leads to bilateral control, and impairments such as mirror movements. However, it has been shown in maturity that strengthening spared ipsilateral CST axons actually leads to functional improvements in a rat model of unilateral CST lesion (46, 70). We think that these findings point to a different culprit. It is not the ipsilateral CST that is maladaptive but rather the consequential loss of the contralateral projection due to reduced ability to compete for synaptic connections in the spinal cord. Furthermore, if the loss of the contralateral projection also triggers afferent fiber sprouting during development, as it does in maturity (48), then there are additional potential sources of maladaptive spinal inputs. It may be necessary to repair multiple neural systems – CST, spinal interneurons, afferent fibers, and rubrospinal fibers, to name four – before motor function can be fully restored. Our interventional approaches that harness neural activity-dependent processes are highly effective in an animal model of hemiplegic CP. These approaches are minimally invasive and are poised for translation to the human.

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Early intervention to improve hand function in hemiplegic cerebral palsy

***Anna Purna Basu*[1,2]*, *Janice Pearse*[1,3], *Susan Kelly*[3], *Vicki Wisher*[3] and *Jill Kisler*[1,2]**

[1] *Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK*
[2] *Department of Paediatric Neurology, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK*
[3] *Paediatric Physical Therapy, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK*

Edited by:
Gavin John Clowry, Newcastle University, UK

Reviewed by:
Jennifer Christy, University of Alabama at Birmingham, USA
Andrea Guzzetta, University of Pisa, Italy

****Correspondence:***
Anna Purna Basu, Institute of Neuroscience, Level 3, Sir James Spence Institute, Newcastle University, Royal Victoria Infirmary, Queen Victoria Road, Newcastle upon Tyne NE1 4LP, UK
e-mail: anna.basu@ncl.ac.uk

Children with hemiplegic cerebral palsy often have marked hand involvement with excessive thumb adduction and flexion and limited active wrist extension from infancy. Post-lesional aberrant plasticity can lead to progressive abnormalities of the developing motor system. Disturbances of somatosensory and visual function and developmental disregard contribute to difficulties with hand use. Progressive soft tissue and bony changes may occur, leading to contractures, which further limit function in a vicious cycle. Early intervention might help to break this cycle, however, the precise nature and appropriateness of the intervention must be carefully considered. Traditional approaches to the hemiplegic upper limb include medications and botulinum toxin injections to manage abnormalities of tone, and surgical interventions. Therapist input, including provision of orthoses, remains a mainstay although many therapies have not been well evaluated. There has been a recent increase in interventions for the hemiplegic upper limb, mostly aimed outside the period of infancy. These include trials of constraint-induced movement therapy (CIMT) and bimanual therapy as well as the use of virtual reality and robot-assisted therapy. In future, non-invasive brain stimulation may be combined with therapy. Interventions under investigation in the infant age group include modified CIMT and action observation therapy. A further approach which may be suited to the infant with thumb-in-palm deformity, but which requires evaluation, is the use of elastic taping. Enhanced cutaneous feedback through mechanical stimulation to the skin provided by the tape during movement has been postulated to modulate ongoing muscle activity. If effective, this would represent a low-cost, safe, widely applicable early intervention.

Keywords: cerebral palsy, early intervention, upper limb, elastic taping, thumb-in-palm deformity, hemiplegia, therapy, orthoses

INTRODUCTION

Cerebral palsy (CP) is the commonest cause of neurological disability in children. The upper limbs are often affected, with significant wrist and hand involvement from an early age (1). Persisting from infancy, affected children may have abnormal hand postures such as thumb adduction and/or flexion with limited wrist extension, as well as more proximal abnormalities of upper limb tone, posture, and function, which also impact on hand use. The particular focus of this article is on the factors influencing hand structure and function in hemiplegic cerebral palsy (HCP), and the options for early intervention.

INTER-RELATIONSHIP BETWEEN BODY STRUCTURE AND FUNCTION, ACTIVITIES AND PARTICIPATION

The international classification of functioning, disability and health (ICF) (2) provides a framework, which describes the inter-relationship between body structure and function, activities and participation, as well as the influence of environmental and personal factors. In the case of hand function in HCP, we see how closely each factor impacts on the others, as discussed below. This can lead to a vicious cycle of deteriorating structure and function and maladaptive activity-dependent plasticity, but can also offer hope for early intervention approaches to break the cycle.

CENTRAL NERVOUS SYSTEM DEFICITS IN HCP

Hemiplegic cerebral palsy affects around 1 in 1300 live births (3) and has a range of etiologies including neuronal migration abnormalities, periventricular leukomalacia, intracranial hemorrhage, and infarction. The common feature is disturbed cerebral control of motor function. A large component relates to corticospinal tract damage, as the corticospinal tract is the major descending tract controlling skilled, fractionated, voluntary hand movements (4). In addition, there is often extrapyramidal involvement (5); some patients have marked dystonia. As well as disruption of motor pathways, there are often sensory impairments including astereognosis (6–9), which impact detrimentally on hand function. These sensory impairments may reflect additional damage to ascending thalamocortical pathways (10, 11) and/or somatosensory cortical areas (9), as well as failure of sensorimotor integration (12). Disturbances of visual function, including but not limited to hemianopia, further contribute to difficulties with hand use (13). Deficits in motor planning and motor imagery as well

as broader deficits in executive function are also seen (14–16). Finally, a minority of patients have significant learning disability, which can also impact adversely on the development of hand function (17, 18).

ROLE OF ACTIVITY-DEPENDENT PLASTICITY WITHIN THE CENTRAL NERVOUS SYSTEM

In addition to structural damage from the initial insult, progressive maladaptive changes occur within the central nervous system due to activity-dependent plasticity. This has been particularly studied within the corticospinal tract (19). Neurophysiological studies in humans indicate that in healthy term infants, corticospinal fibers from each hemisphere project to each side of the spinal cord (bilateral system), with gradual progression to a predominantly crossed projection within the first 2 years of life (20). If this system is perturbed by unilateral perinatal stroke, remaining corticospinal projections from the affected hemisphere may be gradually displaced over time by uncrossed projections from the undamaged hemisphere. This is in general associated with a worse functional outcome than the normal pattern of predominantly crossed corticospinal projections from each hemisphere (21). However, the relationship between corticospinal tract reorganization and functional outcome is complicated, and the nature, timing, and size of the lesion also play a role (22). A marked degree of corticospinal tract reorganization can occur (23).

Progressive maladaptive central nervous system processes in HCP are not limited to the corticospinal tract. Development of spinal cord segmental circuitry is influenced by corticospinal tract activity. If this is disrupted experimentally through transient motor cortex inhibition during early development, subsequent patterns of spinal cord circuitry including interneuron development are abnormal and immature (24–26). In addition, the M1 motor cortical map does not develop normally (27). However, processes such as early restraint of the unaffected limb combined with motor training (28), can reverse to an extent the changes in spinal cord circuitry, corticospinal tract projections, and M1 motor map abnormalities as well as improve motor outcome. Considering this in light of the ICF framework demonstrates that it is possible to alter structure and function either positively or negatively through modulation of activity.

EFFECTS ON BODY STRUCTURE AND FUNCTION

The implications of the above central nervous system changes on upper limb tone, posture, reflexes, and function in congenital hemiplegia are well-known but take time to emerge (17). In the first months of life the typical signs of a hemiplegia are not yet present, although qualitative abnormalities of movement may be detected (29). In addition, many healthy neonates and young infants will demonstrate features such as a thumb-in-palm posture, which disappear over the first few months of life (30). In contrast, the infant developing HCP will often have a closed hand posture and flexed and adducted thumb persisting beyond the first few months.

The development of asymmetrical hand function shows significant variation in infants with HCP; however, pronation of the forearm and thumb abnormalities is most frequently seen (31). As the infant develops reaching and grasping abilities on the unaffected side, parents start to notice a strong hand preference, with lack of use of the affected hand. The fingers are flexed, and the thumb is often adducted and flexed, resulting in the "thumb-in-palm" deformity (32). Dislocation may occur at the metacarpophalangeal joint, with hypermobility at the proximal interphalangeal joints causing swan-neck deformity, which impacts negatively on opposition and grasp (33). The thumb adduction also impacts on grasp, especially pincer and whole hand grasp (34). In a study of children age 4–14 years with CP, marked structural deformities affecting hand function were noted even in the youngest children, without significant age-related increases (1). Another study also reported structural deformities even at age 2 years (31).

More proximal upper limb deficits in hemiplegia also affect hand use. Increased muscle tone is noted predominantly in the upper limb flexor muscles with extensor weakness. Dynamically increased muscle tone is particularly clear in biceps brachii during physical activity such as walking, running, and even moving the dominant hand. Pectoralis major, the forearm flexors, and pronator teres exhibit hypertonia in some children, pulling the shoulder into flexion, adduction, and internal rotation, the forearm into pronation, and the wrist into flexion, often with ulnar deviation. Active forearm supination and wrist extension are limited (32). While some of the increased tone is due to spasticity or rigidity, there are also secondary biomechanical changes in the muscles leading to hypertonus, which is independent of recorded EMG activity, but which is temporarily reduced through muscle stretching ("plasticity") (35, 36). Over time such increased tone can lead to the development of contractures. In addition, there is reduced limb growth on the affected side. Difficulty with selective muscle control and/or excessive co-contraction can discourage use of the hemiplegic limb which in turn compounds muscle weakness (37). Effective bimanual function is often limited (38), with increased reliance on one-handed strategies (39).

EFFECT ON ACTIVITY AND PARTICIPATION

Many daily activities require the hands to perform different movements at the same time in a coordinated way. Generally, the non-dominant hand assists by holding and stabilizing objects while the dominant hand performs more precise functional movements. The child with HCP has to battle with a number of hindrances in order to use the affected hand, and may make an active choice to use the "less-affected" hand alone. [Note that subtle deficits in function are present on the side contralateral to the hemiplegia – hence the term "less-affected hand" (40, 41)]. However, particularly for young children, the reasons for neglecting to use the affected hand are not always clear and the concept of "developmental disregard" has been coined (42). This has been described as the discrepancy between capacity and performance with the affected hand (43). Recent EEG studies indicate an increased cognitive load for movement preparation with the affected hand during a bimanual task (43). This could contribute to a preference for unimanual task performance. Compensatory strategies commonly observed include use of the teeth or stabilizing objects between the knees or against the body instead of using the affected hand. While children may be able to perform some "bimanual" tasks largely one-handed using such strategies, task completion

time often remains prolonged (44). This can lead children to seek the assistance of others, or to avoid certain activities (45). Parents, teachers, and friends may step in too early to provide assistance. Even where the use of both hands is actively encouraged, very young children may not understand why this is important. However, as children get older they begin to recognize things they would like to be able to do more easily, particularly during the teenage years when personal independence becomes increasingly important. Personal and familial emotions and attitudes regarding the hemiplegia will influence for any individual the size of the gap between capacity and performance. These factors are not well described in the literature, though self-esteem and self-concept have been explored (46).

We have illustrated how structural abnormalities in HCP impact on hand function, activity, and participation, and how personal and environmental factors influence this. There is a need for safe, cost-effective, evidence-based early interventions for infants and young children at risk of developing hemiplegia (47). Intervening early could break the vicious spiral of declining structure and function and improve long-term functional outcome. Optimizing hand and wrist posture could encourage the child to experiment more with using the affected limb, drive use-dependent plasticity toward optimal solutions, and reduce the risk of secondary musculoskeletal problems. Consideration should also be made regarding how to enhance visual and sensory awareness of the affected arm and hand and promote its use in play and other activities.

ESTABLISHED MEDICAL AND SURGICAL INTERVENTIONS

Outside the period of infancy, medical and surgical interventions are often considered; these include medications, targeted botulinum toxin treatment, and eventually surgical approaches, with ongoing therapy input and orthoses as appropriate. Botulinum toxin can reduce spasticity in targeted muscles and improve range of movement at a particular joint, facilitating splinting and therapy. This is a time-limited intervention: effects wear off and repeated administration is required. There is concern regarding the long-term effects of repeated injection, resulting in weakness and muscle atrophy in a group of children for whom muscle weakness is already a problematic feature (48). Botulinum toxin is both unlicensed and rarely used in practice for children age <2 years although a recent review of off-label botulinum toxin treatment identified three randomized controlled trials including children in this age range (49). Only one of these was relevant to upper limb function (50), this included children age 22–58 months. The study, while small, indicated a benefit from repeated botulinum toxin injections in the upper limb over and above that of occupational therapy alone, with respect to spasticity and parental perception of performance.

Surgical approaches include tendon transfer, muscle lengthening, and arthrodesis. These well-established procedures offer permanent and fixed solutions: in the case of arthrodesis, the overall range of movement is reduced. Treatment goals include achieving a more functional hand position and improving appearance or hygiene of the arm and hand. Robust studies of long-term outcome are sparse, but there is evidence of benefit (51–53). This includes perceived long-term benefit in terms of function and cosmesis from the patient perspective (54, 55). A full review of medical and surgical interventions is beyond the scope of this article. Below, we focus on therapy and splinting approaches and how these might apply to the infant population.

THERAPY APPROACHES

A number of strategies have been developed aiming to improve hand function in children with established HCP, with varying evidence of benefit (56). Current therapy approaches fundamentally comprise repeated practice of desired movements (sometimes including shaping, i.e., breaking down the goal into incremental steps in line with progress), with the child as an active participant. Most activities consider the principles of motor learning and neuroplasticity and are adapted to the age and cognitive ability of the child (57). Approaches may be play-based, problem-solving, or goal centered, for example, around specific activities of daily living (58). Some approaches such as the use of videogames/virtual reality, or robot-assisted therapy (sometimes in combination with virtual reality games), represent alternative modes of delivery of upper limb therapy rather than radically new principles *per se* (59). In older children with hemiplegia, there are now studies combining non-invasive brain stimulation with occupational therapy approaches.

Some therapies are delivered in intensive bursts (e.g., 60–90 h over a few weeks), which differs from more conventional therapy approaches (57, 60, 61). Information regarding the optimal therapy "dose" is available for only a minority of interventions (62, 63). The practicalities of intensive therapy delivery must be considered in the light of other demands on the child, family, and therapist time as well as other resource pressures. Further information on the relative merits of intensive versus standard models of therapy will emerge in due course (64). A combined approach of infrequent intensive bursts of therapy, with lower-intensity "maintenance" therapy during interim periods, may prove optimal for enhancing and maintaining function.

Of key importance is the transfer of motor function gains from therapy to everyday life, without which there is little point in undertaking the therapy (65). This has been overtly assessed in the adult population in the context of constraint-induced movement therapy (CIMT – see below) (66). Incorporation of a "transfer package" with items such as a behavioral contract, home diary, task practice in the home setting, and ongoing telephone contact beyond the end of the therapy, greatly enhanced the improvements in the amount and quality of arm use compared with lab-based CIMT alone. There is indirect evidence from pediatric studies that similar principles apply (65).

CHALLENGES OF EARLY THERAPY APPROACHES

Developing upper limb therapies suitable for infants is a challenge for many obvious reasons. Infants have short attention spans and little or no understanding of the need for therapy or its aims. This makes it difficult to develop approaches which will be tolerated. Furthermore, evaluating the effects of therapy is tricky in a group with ongoing developmental changes in whom the outcome without intervention can vary. While there is a range of assessments of general motor function in infancy (currently validated to varying degrees), there is only one *validated* assessment

of upper limb function – the Mini Assisting Hand Assessment, which measures function of the affected arm during structured bimanual play in infants 8–18 months (67). A further assessment, the Hand Assessment of Infants (HAI) for measuring unimanual and bimanual function in infants age 3–12 months, is in the late stages of development (68, 69). The grasping and reaching assessment of Brisbane ("GRAB") is being developed for infants from around 3 months (69) but this is also under development and not yet validated.

CONSTRAINT-INDUCED MOVEMENT THERAPY AND BIMANUAL THERAPY

Two therapy approaches, which have received much recent attention, are CIMT, in which the use of the less-affected hand and arm is restricted to encourage use of the more-affected upper limb, and intensive bimanual therapy (62). There is evidence of significant benefit from both of these approaches (56). Bimanual therapy may require a greater intervention duration (62). Guidelines for future research have been drawn up in relation to remaining unknowns regarding constraint therapy, including long-term benefits, effect of repeated programs, effect on bimanual performance, and activities of daily living, optimal type of restraint, type and duration of training, environment where the training is delivered and which patient characteristics influence outcome (70). The long-term effects on function of the less-affected limb must also be monitored (41).

Considering the infant population specifically, there has, to date, been no definitive evaluation of CIMT. However, infants have been included as part of trials studying a broader age group (8 months to 8 years) (71). Similarly, in a non-randomized trial, constraint improved the use of the hemiplegic hand in bimanual play compared with standard therapy approaches in children age 18 months to 4 years (72). The approaches and challenges of CIMT in young children with hemiplegia have been addressed by Taub et al. (73). These challenges are now being addressed in infants at risk of HCP: Eliasson et al. (68) will compare baby constraint-induced movement therapy (Baby-CIMT) with baby massage. Baby-CIMT is described as a modified CIMT protocol, the restraint being a mitt or glove. The intervention will be for 30 min daily for 6 weeks in infants age 3–8 months and will focus on grasping and exploring objects in an enriched environment with close attention to carers' and therapists' behavior toward the infant, toy selection and position of the infant (68). With respect to the need for development of movement control of the "unaffected" hand and for bimanual motor integration in infancy, caution is needed when considering CIMT in infants (41, 70); however, this protocol represents a much reduced intensity of intervention compared with classical CIMT, which would typically involve 60 or 90 h of therapy within a few weeks (63).

ACTION OBSERVATION THERAPY

"Action observation therapy" is a method aimed at stimulating the mirror neuron system – a frontoparietal network of neurons activated both during action observation and action performance. Mirror neurons were first identified by Rizzolatti in animal studies and were so named because they fired when the animal either performed a motor task or observed the same task being performed (74). There is indirect evidence for the existence of a human mirror neuron system (75). Therapies incorporating repeated action observation and imitation showed promise in adults with stroke (76, 77).

Several small studies in children with HCP, generally adopting a bimanual approach in contrast to CIMT, also suggested that a benefit from action observation therapy (78–80). One larger study from our own group (ISRCTN65947097) in children with HCP age 3–10 years has also recently completed. There is to our knowledge only one study exploring the possible benefits of action observation therapy on upper limb function in infants at risk of developing HCP, as well as in healthy control infants (69). The intervention is targeted between the 9th and 13th weeks of life, during which parents will repeatedly demonstrate grasping actions on specific toys. This will be compared with presentations of the toys without demonstrating appropriate grasping actions. The effects on quantity and quality of reaching and grasping, as well as neurophysiological measures, will be assessed. Thus, at present, the role of action observation therapy, though intuitively appealing as a natural way of learning through watching and copying, remains to be established.

HAND SPLINTS/ORTHOSES

A splint has been broadly defined as "a brace, orthosis, cast, tape, or any external device applied to one or more joints" (81). Hand splints may be divided into "non-functional" splints aimed at preventing contracture by providing prolonged stretch, and "functional" splints aimed at improving motor task performance by supporting joints in biomechanically optimal positions (82). Although upper limb orthoses are frequently prescribed by therapists for children with hemiplegia, compliance overall is under 50% (83), with ineffectiveness and refusal to wear the orthosis being reasons for abandonment. Pain, discomfort, and reduction in function have been reported in the adult stroke literature (84). Additionally, by covering part of the palmar surface, thumb, and volar splints reduce palmar sensory feedback, which is vital for sensorimotor integration. All orthoses have the advantage that they can be worn for short periods of time rather than continuously, reducing the risk of disuse atrophy compared with approaches such as casting.

"Non-functional" orthoses offer a benign, temporary solution to correcting posture but are relatively inflexible. This reduces function and with excessive use may lead to muscle atrophy and compensatory overuse of other muscle groups (85). Therefore, non-functional orthoses are often used either at night or for short periods of time to achieve a particular goal, for example, increased muscle length. However, the evidence for this approach (86) or indeed for the benefit of non-functional orthoses (82) is limited. A recent systematic review found five randomized studies of non-functional upper limb splints (82), showing a small immediate benefit for hand function, which was not maintained. The review identified only one study of upper limb functional splinting, in which a Lycra splint from the wrist to the axilla had an additive benefit on goal attainment scores compared with goal-directed training alone (87).

An early description of a functional thumb splint was provided by Currie and Mendiola (88). The splint was assessed in just five

children with HCP, aged 20–26 months. In each child this immediately improved thumb position, grasp type, and spontaneous bimanual exploration. More recently, Louwers et al. (89) studied the immediate effects of wearing a thumb and wrist brace on bimanual hand function in 25 children age 4–11 years with HCP. Performance on the Assisting Hand Assessment (which measures the spontaneous use of the paretic hand to assist in tasks requiring bimanual function) (90) increased by an average of 3.2 raw scores simply by wearing the brace, and 52% of children improved by at least 4 points. No attempt to look at longer-term use, acceptability, or tolerability was made in either of these studies. Ten Berge et al. (91) studied the effects of a 2-month intervention with a neoprene thumb opponens splint on hand function in seven children age 2–7 years with hemiplegia. The splint was prescribed for at least 4 h/day. Compliance was good but goal attainment scores increased above baseline variability in only three patients.

Splints can be particularly challenging for use in young children, who may simply remove them. It is difficult to create well-fitting splints for very small hands and wrists given the nature and thickness of the materials used. Until a clearer evidence base emerges, decisions regarding splinting are fraught with uncertainty (82). In the light of the inadequate evidence base (82), Jackman et al. propose to undertake a study comparing the effects of functional hand splints alone, with task specific training alone, and with both approaches combined, in children age 4–15 years (81).

ELASTIC TAPING

An approach increasingly being used for the upper limb in neurorehabilitation settings (92) but which, to date, has not been adequately evaluated, is the use of elastic taping. This differs from the more rigid forms of "athletic taping" originally developed for sports injuries. Hypoallergenic, waterproof versions of elastic tape are available, as are a range of colors, which can help in attracting the infant's visual attention (though natural, skin tone colors are sometimes more helpful for infants who tend to remove the tape). Specific manuals and training courses are in existence and various methods of application have been described, including a "thumb extension assist" approach for the thumb-in-palm deformity (93). With caution, parents could be trained in application by an appropriately qualified therapist. This is an important consideration because application needs to be repeated frequently (around twice a week) (94).

Many claims have been made regarding the potential benefits of functional elastic taping and these are counterbalanced by both a healthy skepticism and lack of clear evidence of efficacy (95). One of the more plausible stated mechanisms of action relevant to HCP is through enhancement of cutaneous sensory feedback via stretch applied to the skin during movement. For example, taping for the thumb-in-palm deformity includes a longitudinal strip under tension on the dorsum of the thumb, which could lead to increased firing of cutaneous afferents (mechanoreceptors) on the underlying skin during thumb flexion. This could lead to enhanced proprioceptive feedback (96, 97). In fact, skin strain patterns in the hand provide kinesthetic information taking precedence over that from muscle spindle and articular afferents in some situations (98); this kinesthetic role may have been underestimated in the past (99). Complex interactions at spinal cord level lead to integration of signals from the various proprioceptive afferents (100), which can then affect muscle spindle sensitivity through modulation of gamma motor neuron firing (101–103), and ultimately perhaps alter the balance of muscle activity to strengthen thumb extensors over time (104).

This provides the theory; however, the evidence in practice for kinesthetic benefits is lagging. While rigid, athletic tape can stabilize the ankle joint and improve proprioception (105, 106), studies of elastic taping have produced mixed results (107, 108). Additionally, studies in CP as well as in control populations at different ages are essential. This is because cutaneous reflexes show age-dependent changes and are altered in CP. Short-latency cutaneous reflexes are known to be largest in the first year of life, and are also exaggerated in the presence of an upper motor neuron lesion (109). In infants and in CP, stimulation of the digits can produce short-latency excitation of both the forearm flexor and extensor muscles, leading to co-contraction. In the second year of life, long-latency cutaneous reflexes emerge, taking a supraspinal route to the cortex with the efferent volley through the corticospinal tract (109), thus, the long-latency cutaneous reflex is affected in patients with an upper motor neuron lesion. The degree to which patients with CP would demonstrate a kinesthetic advantage from taping is unclear.

Studies in children with CP have tended to focus on practical outcomes such as gross motor development. For example, Iosa et al. (110) studied the effect of taping at the ankle joint in eight children with unilateral spastic CP. Taping was undertaken for 6 months, and led to a greater increment in Gross Motor Function Measure (GMFM) scores (assessed with the tape off) than expected within that timescale, as well as improved gait. The mechanisms behind this were not explored. Interestingly, the only patient who did not improve had marked dyspraxia with sensory integration dysfunction. Similarly, Simsek et al. (111) applied tape for a 12-week period to the paraspinal muscles, aiming to improve sitting posture in children with CP and GMFCS III–V. Fifteen children were randomized to the intervention and 15 were controls. Both groups also received physiotherapy. Sitting Assessment Scale scores (SAS) (112) did not differ at baseline but, were higher in the intervention group (with the tape removed) at 12 weeks. GMFM scores did not improve. Footer also found no benefit of paraspinal muscle taping on GMFM scores over a 12-week period (113).

Elastic taping has been used to improve upper extremity function in adults following stroke, with anecdotal benefit (92). It has also been used for the upper limb both proximally and distally in an acute rehabilitation setting in children with acquired brain injury (114). Children were tested with the Melbourne Assessment of Unilateral Upper Limb Function (MUUL) (115) first with the tape off then with tape applied according to perceived need. Significant improvements were seen both immediately and after 3 days. The range of different taping regimens and the sample heterogeneity pose some problems in interpretation. Mazzone et al. (116) also studied upper limb taping in 16 children with HCP, with a mean age of 3 years. They underwent a 17-month rehabilitation program with tape applied in the first and last 5 months and a 7-month washout period in the middle. Taping included the thumb and spiraled up the arm to the middle third of the humerus. The results were suggestive of improvement only in the

taped periods, with the caveats that dropout rate was high (50%) and the assessment used (MUUL) was not suitable for children aged below 5 years.

We are unaware of any other published literature on the effectiveness of elastic tape in improving hand and wrist position and function in infants with CP. As it is increasingly being used in practice, a formal evaluation would be timely to determine if children with thumb-in-palm deformity benefit from this intervention, and if so, how.

CONCLUSION

Early intervention for the upper limb in hemiplegia remains challenging, though progress is being made. We have touched earlier on the difficulties of outcome assessment in the youngest infants and children, which make evaluation of interventions very difficult. The diversity of the population under study, in terms of lesion type, differences in post-lesional reorganization, and the degree to which other factors such as vision, sensation, and cognitive ability impact on hand function, must also be considered. Studies tend to be small and are dominated by short-term outcomes (with the aim of avoiding confounders due to developmental trajectories), whereas the long-term outcome may be the more important consideration. Therapy approaches including CIMT and action observation therapy are now being actively explored in the youngest children and infants. Functional elastic taping has some potential merits in this age group but requires further investigation with properly controlled studies. These should include assessments which could shed light on the underlying mode of action.

AUTHOR CONTRIBUTIONS

Anna Purna Basu drafted the manuscript, to which Janice Pearse, Susan Kelly, Vicki Wisher, and Jill Kisler contributed substantially. All authors have seen, agree to, and are prepared to be accountable for the final version of the manuscript.

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