CHAPTER 1: EXECUTIVE SUMMARY

The California Institute for Regenerative Medicine (CIRM) is charged with supporting the development of cures and therapies for human disease, based on stem cell science. This mission necessarily encompasses a wide range of research activities, from basic to clinical, in every area of human biology. The mission is particularly challenging given that the applicability of stem cell therapies varies depending on the disease, on our understanding of the clinical pathophysiology underlying it, and on our knowledge of the biological mechanisms governing the tissues involved. CIRM periodically organizes workshops to evaluate the impact of stem cell technologies on particular fields or disorders and to determine how to target CIRM resources to maximize this impact. In June 2011, a CIRM-sponsored workshop was undertaken to review our current understanding of the neurobiological defects underlying Cerebral Palsy (CP) and to identify emerging areas of therapeutic development for these conditions. One goal of this workshop was to explore the role that stem cell research might have on the development of therapies for these conditions. Participants were asked to recommend a course of action that CIRM might take to advance translational activities in this field.

Neurobiological disorders of childhood (NDCs), such as CP and autism, are common and devastating. They also have severe social and economic impact on individuals and families throughout the lifetime of the patients. CIRM has identified NDCs as an important area for stem cell research because 1) these disorders are relatively understudied, in part due to restrictions on research in children; 2) pioneering research suggests that these disorders are amenable to ***in vitro*** **studies** using human stem cells and induced pluripotent stem cells (iPSCs); and 3) these disorders are potentially good first targets for **cell therapy** in the brain, since therapy could be attempted early in development when the brain presents a more receptive environment for engraftment. There are many features of CP that make it a particularly attractive target for therapy-directed stem cell research. First, as a general disease involving demyelination, insight into the pathogenesis of CP might have important implications for understanding other demyelinating diseases, including multiple sclerosis (MS), the most common cause of neurological dysfunction in young adults worldwide. Other common neurological diseases, like stroke, could also be impacted through in depth studies of CP. Developing *in vitro* models of CP could impact our understanding of the biological mechanisms underlying normal development and of the processes involved in several different brain disorders. Second, since the damage observed in CP is regionally restricted, non-progressive, and appears limited to a few cell types, stem cell therapy has tremendous potential. Investing in basic animal models for CP that focus on improving diagnostics as well as treatment regimens could feed directly into improving outcomes for all cell therapies for other demyelinating diseases (Goldman et al., 2011). The experimental control offered by existing animal models of CP could be an excellent testing platform to advance the development of therapies for other neurological disorders.

In spite of the obvious synergistic benefits of pursuing research in stem cell and regenerative medicine for childhood disorders of the brain such as CP, funding has been limited for projects in these areas.. The clear conclusion of this Workshop was that the science is at a stage where CIRM and other funding agencies can provide meaningful support to stem cell research on childhood brain disorders. In the final chapter of this report, CIRM offers a proposal to stimulate research in these areas.

CIRM WORKSHOP: EVALUATING CP

World leading specialists in CP including pediatric neurologists and other practicing physicians, basic scientists and clinical researchers, together with patient advocates from overseas and from the CIRM Board, were invited to San Francisco to discuss with CIRM the status of CP research. Leading clinicians and scientists were asked to evaluate ongoing clinical and basic research efforts in the field and identify challenges to therapeutic development. Importantly, they considered whether research using stem cells would advance our understanding of the biology of CP and lead to conceptual or therapeutic breakthroughs.

MOVING FORWARD: STEM CELLS AND CP

Stem cell studies could contribute to the development of cures and therapies for CP in three different ways:

1. Endogenous stem cells and animal models: CP involves neuronal and glial lesions of the developing nervous system. Studying the processes underlying normal brain development will help us understand the effect of lesions and other disruptions on the structure and function of the mature brain. In addition, it could help us develop interventions that protect surviving cells and stimulate endogenous stem cells to replace the damaged tissue.

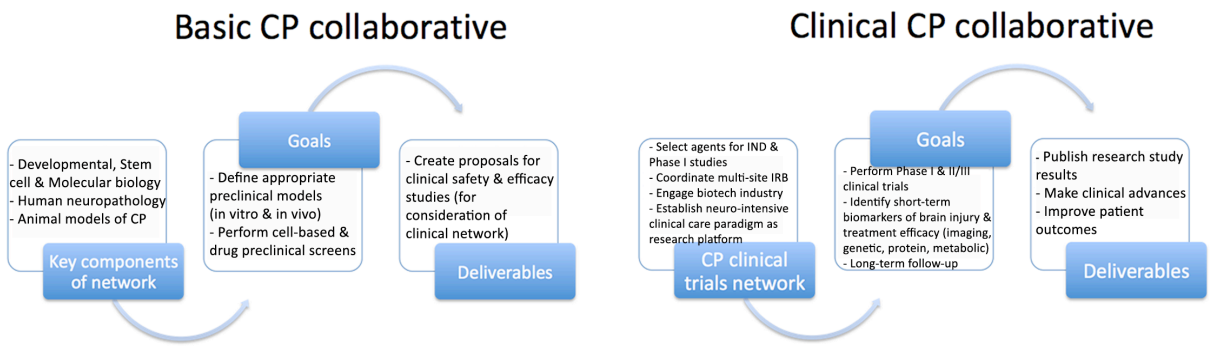
2. *In vitro* human models of CP: Differences between animal and human cells could be one reason for the high failure rate of current drug discovery paradigms in the brain. Induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) can be used to study the differentiation and function of *human* neurons and glia, as opposed to animal cells. Furthermore, by using cultures containing both neurons and glia, researchers can study network processes such as myelination or the development of synaptic connections at the molecular level. These models could be used to better understand the effect of damage on both cell-intrinsic features such as differentiation and cell-extrinsic processes such as myelination in more complex co-culture models. Finally, researchers could use iPSCs from patients to characterize the cellular basis of any genetic susceptibility to CP, which might help in customizing therapies to particular groups of patients. Such use of patient iPSCs is sometimes referred to as a "clinical trial-in-a-dish" approach.

3. Cell replacement therapies: Since the lesions underlying CP occur during *in utero* and early postnatal development, at a stage when the brain is normally integrating new cells into a developing network, cell replacement therapies might be particularly effective for CP. Research in this area is still in its infancy: aspects such as the appropriate cell type to be transplanted or the precise timing of transplantation have not been determined. Researchers will need to develop more sophisticated neuronal and glial differentiation protocols, define the progression of the lesions and the optimal time after lesion for attempting a regenerative therapy, and study the effectiveness of cell transplantation in reversing CP in animal models before moving into human patients.

CP is a heterogeneous group of disorders with diverse etiology. Workshop participants proposed a framework for the type of basic and clinical questions that should be addressed to understand the effects of injury on development, involving two collaborative efforts by basic and clinical scientists (Figure 1). Basic scientists need to continue studying normal brain development and define the factors required for the differentiation and regeneration of oligodendrocytes and other cell types damaged in CP. Importantly, this research needs to be validated in humans. In addition, existing animal models need to be optimized for testing the safety and efficacy of potential stem-cell-based therapies for

reversing the effects of CP, as outlined in "Basic CP collaborative" (Figure 1, top). These models might also be useful for developing tools to monitor brain lesions and to reduce the effects of inflammation. Finally, *in vitro* models that accurately mimic *in vivo* processes such as myelination need to be developed using human cells. Clinical researchers need to continue characterizing the damage that is relevant to the underlying symptoms (for instance, studying the functional relevance of micro- or macrolesions), more carefully identify the critical period of injury and the ideal timing for therapeutic intervention, and develop short-term biomarkers for evaluating disease progression and the effect of potential therapeutics (see "Clinical CP collaborative", Figure 1, bottom). This comprehensive approach would not only maximize the chances of developing stem-cell-based therapies for CP, but could lead to breakthroughs in our understanding of human development that will help us evaluate and treat other NDCs and adult brain disorders.

Figure 1. Proposed organizational structure of the Basic and Clinical CP research collaboratives.



WORKSHOP OUTCOMES

Workshop participants were enthusiastic about the concept of CIRM increasing its support of CP research specifically, and of early brain development and NDCs in general. They proposed the following goals:

- To raise awareness of CP and other NDCs among stem cell scientists;
- To encourage the stem cell community to expand its studies on neural and glial development, the effect of injury and timing of injury on these processes, and other CP-related issues, particularly in humans;
- To provide funding for basic and clinical scientists working on CP and related disorders of the developing brain, particularly in human;
- To encourage the formation of clinical and basic science partnerships working towards specific therapy-based deliverables (see Figure 1).

To achieve these goals, workshop participants indicated that CIRM should clearly identify human neural development and developmental disorders of the brain, including CP, as priorities in its existing RFAs. In addition, CIRM should aggressively promote research on neurobiological disorders of childhood. Finally, CIRM should help promote the formation of Basic Science and Clinical Research collaborations for CP. Workshop participants agreed that this three-pronged approach would have the greatest impact on therapeutic development for CP and related brain disorders.

CONCLUSION

Studying stem cells will further our understanding of early development in humans and of some of the processes that might be disrupted in CP. It could also have a broader impact on NDCs and on other disorders of the brain, particularly PMD and MS. Although there is not at this moment a clear path to the clinic for stem cell-based therapies for CP, CIRM was seen as instrumental to facilitating research and therapeutic development for childhood neurobiological disorders through its funding and its leadership in translational science. Such efforts would position California as a world leader in research and potential new therapies for this important class of disorders.

CHAPTER 2: CLINICAL OVERVIEW AND THERAPEUTIC DEVELOPMENT

In the 1860s, English surgeon William Little described a non-progressive neurological condition in children causing stiff, spastic muscles in their extremities, and attributed the condition to obstetrical complications at birth (Little, 1861). Seventeen years later, Sigmund Freud noted that spastic diplegia (as the condition described by Little came to be known) and other forms of cerebral palsy (CP) were associated with diverse neurological deficits, and proposed that the condition was more likely induced by disturbances during fetal development (Obladen, 2011). However, this hypothesis was not confirmed until the 1980s, when a study of 35,000 patients sponsored by the National Institutes of Neurological Disorders and Stroke (NINDS) confirmed that the majority of cases of CP were not associated with complications during labor but rather seemed to result from earlier insults to the brain (From the Task Force on Joint Assessment of Prenatal and Perinatal Factors Associated with Brain, 1985; Nelson and Chang, 2008). Publication of this study led to an explosion of clinical studies aimed at describing the pathology and etiology of CP.

CP is currently understood as a disorder induced by injury to the brain during late prenatal and early postnatal development. The principal pathological observations are lesions to the white matter and degeneration of oligodendrocytes, the myelinating cells of the central nervous system (CNS). In addition, there is some thought that astrocyte hyperproliferation, local inflammation, and failures in axonal regeneration may also contribute to disease physiology (Alvarez-Diaz et al., 2007; Volpe, 2009a; Silbereis et al., 2010). In this section, we provide a general overview of the clinical features observed in patients, and the neuropathology involved.

A. Clinical definition, etiology, and definition of terms

The opening session of the Workshop, provided an overview of the clinical characteristics of the disorders grouped under the umbrella term “cerebral palsy.” Given the varied etiology and symptoms that are involved, participants thought it important to define the diseases more specifically, in ways that were relevant to CIRM’s goal of developing therapies based on stem cell research. The consensus definitions described below, developed by Stephen Back and David Rowitch with modifications from the participants, will be used throughout the accompanying workshop report.

Definition of Cerebral Palsy (CP): CP denotes a condition of the brain reflected by movement limitation that is often associated with some degree of cognitive impairment, and disturbances of sensation, communication, perception, or behavior. While it is generally considered a fixed, non-progressive condition resulting from neurological injury in the antenatal or perinatal period, there is good evidence of metabolic, histological, structural, and behavioral evolution of CP over a surprisingly prolonged period of early childhood. Children grow into their deficits, and this phenomenon suggests that there exists times to intervene to promote functional connectivity in central nervous system circuits.

Cause of injury and underlying etiology: Although it is generally agreed that CP arises from brain injury during development, the prenatal and/or perinatal circumstances giving rise to injury in individual patients is varied and often mysterious. Conditions associated with acquired brain injuries that can lead to CP include the following (Rezaie and Dean, 2002; Silbereis et al., 2010):

1. *in utero* events (such as hypoxic-ischemic encephalopathy (HIE) caused by placental insufficiency, chronic fetal-to-maternal

hemorrhage or stroke, as well as infection, inflammation, and exposure to combined insults;
2. interrupted brain development due to premature birth (defined as birth before 37 completed weeks of gestation);
3. perinatal events (such as placental abruption or respiratory failure, stroke, infection);
4. neonatal disorders (such as chronic lung disease);
5. genetic disorders (such as col4a1 mutations causing porencephaly)

The diverse etiology of CP results in heterogeneous lesions in different regions of the brain, as discussed in further detail below.

It is known that insults leading to CP can occur early, during weeks 24-32 of gestation (Volpe, 2009a), a critical gestational period during which the brain is undergoing the early stages of neural development. This is a highly complex process that we are only beginning to understand in humans. Thousands of cell types, including neurons and glia, are born and are directed to form an interconnected network through a combination of genetic and environmental processes. Disruptions to these processes due to physical injury or to chromosomal defects can lead to delays in neuro- and gliogenesis, and can have profound and long-term effects on the generation of new cells and the formation of neural networks essential for cognitive and motor function.

Definition of White Matter Injury (WMI): Injury to the periventricular white matter (often called periventricular leukomalacia) is one of the most common structural defects in CP. For the purposes of this report, WMI is defined as a spectrum of pathology that includes: (1) the classic lesion of periventricular leukomalacia (PVL), which involves macroscopic cystic or microscopic non-cystic necrotic lesions with pan-cellular degeneration, and (2) focal or diffuse non-cystic lesions. These lesions may selectively trigger oligodendrocyte lineage degeneration or maturation arrest and subsequent disturbances in myelination. Neuronal loss and axonal damage are often observed in patients with WMI; these events can reflect primary injury or arise as a secondary response to WMI. However, the extent and functional impact of neuronal loss and axonal degeneration in the non-cystic lesions that predominate in most patients remain to be determined.

Other Injuries in Pre-term Infants Associated with CP:
Global loss of brain tissue in cortex (resulting in ex vacuo hydrocephalus) and /or cerebellum is a common finding in children born preterm that are at risk to develop CP (Volpe, 2009). Although the etiology remains obscure, recent studies highlight the association of postnatal gluco-corticoid administration and cerebellar hypoplasia (Tam et al., 2011) through inhibition of Sonic hedgehog signaling (Heine et al., 2009; Heine et al., 2011).

Injuries in Term Infants Associated with CP:
Hypoxic-ischemic injury occurs in 1 in 2000 live births and stroke occurs in 1 in 4000 live births (Boniacio et al, 2011). Both of these injuries result in multi-system dysfunction that can result in lifelong cognitive disabilities, motor impairments, epilepsy and behavioral disorders.

Symptoms and clinical characteristics: Emily Tam and other clinicians attending the Workshop provided detailed descriptions of the symptoms of CP, which include ataxia, spasticity, tremors, paralysis, awkward posture or gait, variations in muscle tone, and difficulty with precise motions. Patients are categorized by severity into five levels – ranging from patients who can walk (Level I) to patients who must be transported in a wheelchair (Level V) (Palisano et al., 1997) (Table 1). Importantly, physicians indicated that the nature of the injury and its timing affect the symptoms and their severity among individual patients (Ferriero, 2004) (Figure 2). Finally, speakers indicated that most cases of CP are associated with some

degree of cognitive impairment and disturbances of sensation, communication, perception, or behavior that also need to be considered during therapeutic development (O'Shea, 2008; Miller and Ferriero, 2009; Volpe, 2009b, a; McCormick et al., 2011).

Disease Progression: CP is generally considered to be a fixed, non-progressive condition once the initial damage has been consolidated. However, physicians and basic scientists agree that the injury site evolves in response to ongoing neurobiological development such that there are metabolic, histological, structural and behavioral changes as the infant brain matures through early childhood (O'Shea, 2008). In addition, Donna Ferriero pointed out that timing matters in brain injury such that similar insults can result in different clinical patterns depending on when in development the injury occurs. This also results in different signs and symptoms emerging only as the children mature and are unable to reach developmental milestones (Ferriero, 2004). CP is therefore experienced as a neurodevelopmental disorder by patients and caregivers, with symptoms arising as the child matures physically and cognitively.

Table 1. Summary of the *Gross Motor Classification System for Cerebral Palsy*. Adapted from Palisano et al., 1997.

Years	Level I	Level II	Level III	Level IV	Level V
0	- Able to sit up and balance while manipulating objects with both hands - Crawls on hands and knees - Begins to walk between 1.5-2yrs without assistance	- Maintains floor sitting but needs to use hands for support to maintain balance - Creeps on stomach or crawls on hands and knees - Pulls to stand & take steps while holding furniture	- Maintains floor sitting while low back is supported - Rolls and creeps around on stomach	- Trunk support required for floor sitting - Can roll to supine and may roll to prone positions	- Unable to maintain antigravity head and trunk postures while prone and sitting - Requires assistance to roll
2	- Able to move in and out of floor sitting positions and stand without assistance - Walks as preferred method of mobility without assistive devices	- Maintains floor sitting but has difficulty with balance when both hands occupied - Crawls, pulls to stand on stable surface & walks short distances with assistive devices	- Requires assistance to assume floor sitting, can maintain by "W sitting" - Crawl on hands & knees, move using stable surfaces & walks short distances with assistive devices, adult assistance	- Floor sit when placed but unable to maintain balance & alignment without using hands for support - Require mobility devices for sitting and standing, short-distance mobility achieved through rolling, creeping on stomach, crawling	- Voluntary control of movement and all areas of motor function restricted - No means of independent mobility; require power wheelchair with extensive adaptations throughout life
4	- Moves from floor to standing to sitting on chairs without stable external support - Walks indoors & outdoors and climbs stairs - Beginning to run and jump	- Sits in chair with both hands free but requires stable external support to move from floor or chair sitting to standing - Walks short distances without assistive devices - Unable to run and jump	- Sit on chair with trunk support to maximize hand function - Walks short distances with assisted mobility devices	- Sits on chair with adaptive seating & requires assistance moving into and out of chair - Walks short distances only with assisted mobility devices & adult assistance - Long distance mobility requires power wheelchair	
6	- Walks indoors and outdoors and climbs stairs without limitations - Can run and jump but speed, balance, coordination reduced	- Walks on even surfaces and climbs stairs but experiences difficulty with uneven surfaces, inclines - Ability to run and jump is minimal at best	- Walks on even surfaces with assisted mobility device - Mobile over long distances with manual or power wheelchair depending on upper limb function	- Maintains function achieved before age 6 - Relies on power wheelchair for self-mobility	
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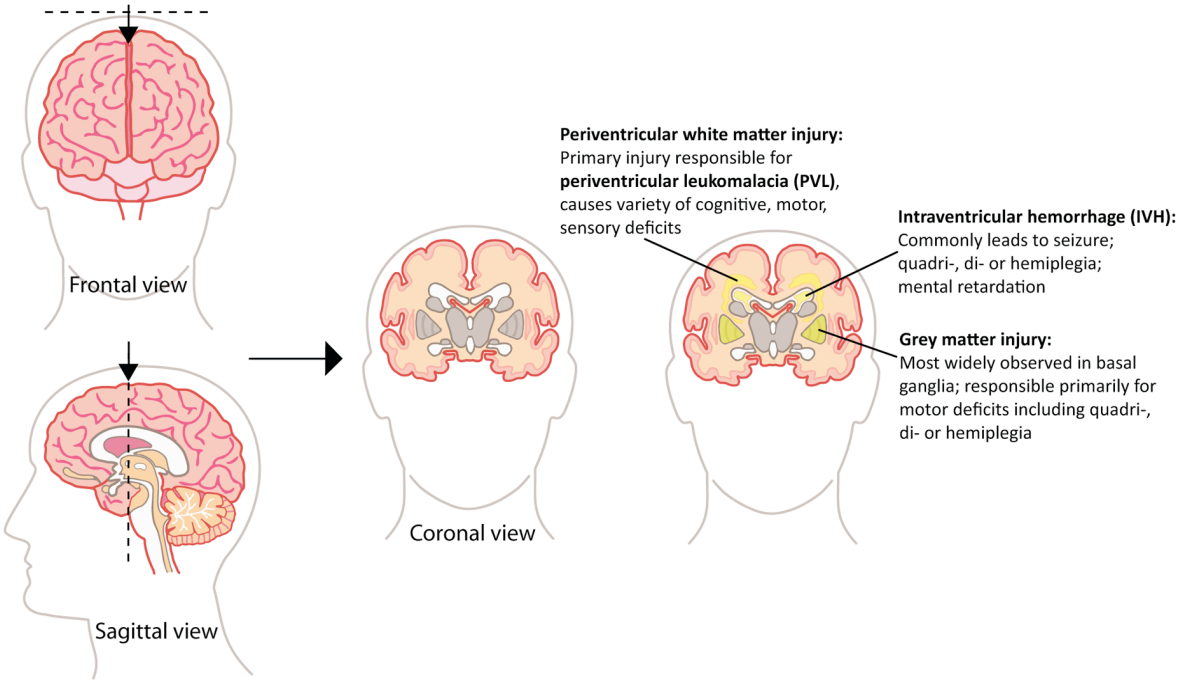
Figure 2. Examples of common symptoms observed in CP patients with respect to the lesion site. Far left panel shows the frontal (top) and sagittal (bottom) views of the brain, that when sliced in coronal section (center) reveals typical locations of injury and their symptoms (far right).



B. Disease impact

CP is a tragically common condition that adversely affects the quality of life of patients and caregivers. The NIH has estimated that CP affects 800,000 patients in the United States alone. It has an incidence of 1-2.5 per 1000 births (Paneth et al., 2006; O'Shea, 2008), with the highest rates observed in premature infants born before the 37th week of gestation (Ferriero, 2004; Volpe, 2009a, b; Silbereis et al., 2010). Steve Back indicated that between 5 and 10% of infants born prematurely go on to develop CP, whereas 25-50% have more subtle cognitive defects (Volpe, 2009b). In spite of increased research and significant improvements in care, the prevalence of CP has not decreased over the last three decades. Rather, it has increased in recent decades probably because of increased rates of prematurity and survival of extremely low birth weight premature infants that are at highest risk of neurological injury (Moster et al., 2008; Silbereis et al., 2010). As is the case with many neurobiological disorders of childhood, individuals with CP have a relatively long life expectancy and because their symptoms do not improve with age, the economic impact of these disorders is severe. The lifetime cost per CP patient in the US was estimated at nearly $1 million in 2003 (CDC, 2004), and Australia estimated that the financial impact of CP in 2007 was around 0.14% of its GDP or $1.47 billion dollars (AccessEconomics, 2008). Nadia Badawi from the Australian Cerebral Palsy Alliance stressed during the Workshop that there is an urgent medical and societal need to develop therapeutic options for these disorders.

C. Neuroanatomical defects and brain imaging

At birth, the human brain consists of billions of interconnected neurons and glia and has all the gross morphological features that can be identified in the adult. This prenatal development is completed in the remarkably brief 40 weeks following conception. By 18 weeks of gestation, patterning has already been established in the brainstem and cortex; by 26 weeks the sulci can be identified as involutions; and tertiary gyri are observed by week 32 (Barnette et al., 2009; Lodygensky et al., 2010). Given its speed and complexity, it is not surprising that the process of neural development is very susceptible to lesions and damage.

The pathological characteristics of developmental brain injury have been studied using both classic neuroanatomical techniques on brain sections and more modern imaging tools such as Magnetic Resonance Imaging (MRI). These studies have revealed both focal and diffuse damage to white and gray matter in patients with CP. Hannah Glass showed typical MRI findings of the common defects include a marked reduction in the size of the corpus callosum and dilation of lateral ventricles with WMI, and basal ganglia and vascular watershed lesions with grey matter injury, but damage is heterogeneous in both the type and location of injury (Inder et al., 2005). In studies correlating the pathological observations with patient phenotypes, white matter injury (WMI) is often observed in spastic diplegia and quadriplegia; deep gray matter injury correlates with dyskinetic movements; and hemorrhage can lead to all of these disorders and also to mental retardation and seizures (Rezaie and Dean, 2002; Mercuri and Barnett, 2003; Miller and Ferriero, 2009; Volpe, 2009b; Stoinska and Gadzinowski, 2011) (Figure 2).

Eric Huang presented neuropathological findings for some of the common developmental brain injuries. Neuropathology differs with etiology and timing of injury (Ferriero, 2004). Injury due to hypoxic-ischemic encephalopathy (HIE) in the term newborn can result from number of pre- and perinatal events in including placental abruption, stroke, and infection. . It is characterized by infiltration of glial cells to the site of injury, which may contribute to damage (Rezaie and Dean, 2002; Volpe,

2009b). Severe HIE can result in loss of cortical and basal ganglia neurons with profound astroglial scar. CP with preterm etiology is linked most strongly to WMI and associated damage. Myelination defects are clearly observed in periventricular leukomalacia (PVL) (Rezaie and Dean, 2002; Folkerth, 2005; Vope, 2009b; Silbereis et al., 2010). In cases of gray matter injury, numerous ischemic neurons are observed in the striatum and affected regions suffer loss of neurons (Rezaie and Dean 2002; Volpe, 2009b). Overall CP in babies born preterm correlates most strongly with injury in the periventricular white matter, gliosis, and degeneration of myelin-producing oligodendrocytes. Little axonal degeneration has been observed, as axons seem to be preserved early in disease progression (Banker and Larroche, 1962; Pierson et al., 2007). Lesions include the focal cystic lesions seen in PVL, non-cystic focal gliosis and necrosis, and diffuse disturbances in myelination (Volpe, 2009b, a). Neuropathological and MRI analysis of macroscopic PVL lesions reveals extensive local reaction to injury, with large focal cystic necrosis and areas of pallor that have oligodendrocyte degeneration and accumulation of reactive astrocytes and microglia. Almost all patients with macroscopic PVL develop CP. However, Stephen Back indicated that over the past 15 years, there has been a dramatic decline in the burden and the incidence of macroscopic focal necrosis and, when it occurs, a decrease in the extent of the damage as observed principally by MRI (from 54% in 1983-2000 to 34% in 2003-2010). Improvements in medical care have led to a shift from macroscopic focal cystic lesions, which now represent only 8% of cases, to milder and more diffuse forms of injury (Folkerth, 2005; Volpe, 2009b). Contemporary cohorts present principally with noncystic focal gliosis or diffuse microscopic necrosis (Folkerth, 2005; Volpe, 2009b). Injuries can present with axonotomy and other evidence of gray matter degeneration, although these are observed more commonly in cases of focal and diffuse injury (Folkerth, 2005; Silbereis et al., 2010). The cause of this axonotomy is not fully understood, although it is linked to the acute inflammatory environment caused by the injury (Folkerth, 2005).

MRI analysis of the lesions in both preterm and term newborns has led to the identification of features in the internal capsule, basal ganglia, and cortex that predict CP (Sie et al., 2000; Ferriero, 2004). Hannah Glass indicated that these MRI features seen early might potentially be used prognostically as well as diagnostically.

Adam Kirton described the common imaging findings seen with perinatal stroke, including arterial ischemic and venous thrombotic events. He also described the phenomenon of presumed perinatal stroke, a condition where the baby is not identified until infancy, and imaging findings suggest that the etiologic pattern is consistent with a vascular stroke occurring at or near birth (Kirton et al, 2008).

D. Developmental and cell biological defects in patients

The most challenging barrier to therapeutic development for CP is our incomplete understanding of the mechanisms that underlie early brain development, and of the disease processes triggered by injury during this critical period. David Rowitch chaired a session that proposed a greater understanding of human brain development could provide critical perspective for understanding root causes of injuries leading to CP as well as ways to better marshall the brain's native repair capabilities. Current evidence suggests that CP in preterm infants prominently affects myelinating oligodendrocyte populations of the brain, and neuroanatomical studies presented at the workshop emphasized the oligodendrocyte contribution to CP. However, more research on the cellular and molecular pathophysiology underlying CP is essential to developing effective treatments.

Oligodendrocytes (OLs) insulate axons by ensheathing them in multiple layers of myelin, which is critical for the rapid conduction of electrical currents in neurons (Franklin and ffrench-Constant, 2008). OLs are generated by sequential differentiation of neural stem cells, oligodendrocyte progenitors (OLPs), pre-myelinating oligodendrocytes (pre-OLs) and finally myelinating OLs. In humans, OLP are produced in high numbers in third trimester and postnatally (Rowitch, 2004). They also appear to be vulnerable to hypoxic-ischemic damage of the sort that is seen following neonatal brain injuries (Alvarez-Diaz et al., 2007; Volpe, 2009a; Silbereis et al., 2010). Although this notion is challenged by several recent studies. In WMI-induced forms of CP, death and reactive hyperproliferation of pre-OLs, followed by an apparent developmental arrest, result in very low numbers of mature OLs (Alvarez-Diaz et al., 2007; Volpe, 2009b; Billards et al, 2008; Buser et al., 2011; Silbereis et al., 2010). The resulting absence of myelin dramatically slows down neurotransmission, and if prolonged is thought to trigger axonal degeneration (Fancy et al., 2011a). David Rowitch described how dysregulation of Wnt signaling is active in neonatal WMI and that Wnt activity results in blocked differentiation of OLPs. Moreover, a drug reversing Wnt effects accelerates repair of demyelinated lesions (Fancy et al., 2011). Vittorio Gallo's work shows that hypoxia induced reduction of p27 leading to enhanced OLP proliferation but diminished differentiation and myelination following hypoxia. Further identification of critical mechanisms responsible for OLP differentiation block in neonatal WMI is an important step to development of novel rational therapies.

The OL damage typically observed in CP is sustained during a critical developmental period of rapid neural growth and maturation, and causes an irreversible interruption of the normal patterning and development of synapses and myelinated neuronal tracts within the injury site. CP thus appears to be caused in part by an impact on developing oligodendrocytes and related failures in axonal function during antenatal or early postnatal development. Major contributors to OL damage following neonatal injury include glutamatergic excitotoxicity due to hypoxia-ischemia, free radical and cytokine-mediated injury triggered by reactive microglia, a developmental lack of oxidative enzymes that normally regulate oxidative stress, and astrocyte-mediated gliosis (Rezaie and Dean, 2002). Many of these involve cell-extrinsic effects, and Stephen Back presented MRI and neuropathological data supporting the hypothesis that WMI lesions involve complex effects of reactive astrogliosis and microgliosis on the OL lineage. David Rowitch pointed out neuropathological similarities of lesions of CP with MS, in which a block of OL differentiation is also proposed to result in chronic demyelinated areas of the brain.

Although much of the research into mechanisms of neonatal brain injury is correctly focused on oligodendrocytes, it is important to stress that other cell types also contribute to the pathology of CP. Astrocytes are found throughout the CNS and are a critical constituent of the blood brain barrier. Under normal physiological conditions, astrocytes provide metabolic support to neurons, regulate the extracellular ionic environment, and clear excess neurotransmitters from the extracellular space (Rowitch and Kriegstein, 2010). In conditions of brain injury, astrocyte proliferation promotes repair both by filling in the damage site (astrogliosis) and by releasing neuroprotective factors including erythropoietin and HIF1A (Liu et al., 2006; Alvarez-Diaz et al., 2007). On the other hand, Jennifer Zamanian indicated that astrocytes also produce free radicals and pro-inflammatory cytokines following injury, both of which are harmful to oligodendrocytes and consequently impede remyelination (Deguchi et al., 1997; Rezaie and Dean, 2002; Villapol et al., 2008). Thus, astrocytes promote repair but also

contribute to the lasting damage to the OL lineage that underlies CP brain injury.

Microglia are the resident macrophages of the CNS. Their job is to scavenge and remove harmful debris from the CNS, and they therefore represent the main effector immune cells involved in the response to neuronal injury. Following insult, microglia infiltrate the damage site and promote repair by quickly eliminating the cellular debris that can cause cell death and damage (Alvarez-Diaz et al., 2007). However, activated microglia also release pro-inflammatory factors such as TNF-α and other pro-inflammatory cytokines that are highly toxic to oligodendrocytes (Bessis et al., 2007; Leviton and Gressens, 2007). Thus, microglia share with astrocytes the distinction of both promoting neuronal repair and inhibiting full restoration of the injury site by inhibiting remyelination.

Pierre Gressens and other workshop participants stressed that although glial damage is the predominant pathological observation, neuronal injury could still be a major contributor to the deficits observed in CP (Dommergues et al., 2000; Leviton and Gressens, 2007; Pierson et al., 2007). Neonatal injury, and hypoxic-ischemic encephalopathy (HIE) in particular, can lead to lasting axonal disruptions in humans. Although neuronal death is often minimal in most forms of CP, the loss of even small proportions of neurons could disrupt the formation and function of neuronal circuits, which could have powerful effects on cognitive and motor function. This hypothesis is supported by observations that axon diameter is reduced in some rodent models of perinatal systemic inflammation (Favrais et al., 2011). Further basic and clinical research is needed to assess the involvement of axonopathy and neuronal dysfunction in CP.

As indicated above, there is a significant increase in CP in preterm infants. Anna Penn provided intriguing evidence that some of this increase could be due to the loss of a placental supply of neuroactive peptides and steroids at a critical time in development. Neurosteroids in particular cross the fetal blood brain barrier and have neuroprotective effects during normal development (Hirst et al., 2009). They might also affect the proliferation of glial cells and neural stem cells (Wang et al., 2005), although more research is needed to support this hypothesis. Dr. Penn indicated that Phase II clinical trials are underway to test the effectiveness of administering synthetic neurosteroids to adults with epilepsy and to infants who have infantile spasms, opening the possibility of using neurosteroids to protect and increase neural and glial stem cell proliferation in vulnerable preterm newborns.

E. Current trials and interventions for neurobiological disorders of childhood

Victorio Gallo chaired the session directed toward new clinical approaches. Fernando Gonzalez provided excellent overviews of the intervention strategies being tested clinically for CP. The only approved clinical intervention for hypoxic-ischemic encephalopathy (HIE) is therapeutic hypothermia, which if initiated within the first six hours of life has been demonstrated to significantly reduce mortality as well as long-term neurodevelopmental disability (Gluckman et al., 2005; Shankaran et al., 2005; Gonzales and Ferriero, 2009). There is evidence that hypothermia decreases the release of glutamate and of oxygen and nitrogen free radicals following injury, reduces apoptosis, and prevents the later stages of energy failure observed following HIE.

Although hypothermia protects cells from cell death, it does not have neurogenic or gliogenic effects. Other promising pharmacological treatments for brain injury include support with trophic factors such as erythropoietin (EPO) and vascular endothelial growth factor (VEGF), which in animal models have both been shown to enhance neurogenesis and promote

neuroprotection (Jin et al., 2002; Mu et al., 2003; Gonzalez et al., 2007; Gonzalez and Ferriero, 2009; Gonzalez et al., 2009; Iwai et al., 2010). Administration of EPO early in the progression of the injury leads to a short-term improvement in hemispheric volume and sensorimotor effects, and long-term improvements in cognition (Chang et al., 2005; Sola et al., 2008). Administration for longer periods following injury leads to increases in oligodendrocyte differentiation and function without changing lesion size (Iwai et al., 2010). Trials assessing the neuroprotective benefits of EPO in human neonates are ongoing (trial #NCT00910234, www.clinicaltrials.gov), and Dr. Gonzalez indicated that to date the treatment appears safe and potentially efficacious. Therapies that decrease excitotoxicity and oxidative stress might also be promising for addressing the vulnerability of the developing brain to excitotoxic and oxidative stress.

Anna Penn suggested that neuroactive steroids might provide a novel therapeutic strategy for WMI of the preterm, as discussed above. Vittorio Gallo showed data demonstrating oligodendrocyte regeneration after prolonged perinatal hypoxia, and the potential avenues for therapeusis that this model suggests.

Finally, Charles Cotton and Joanne Kurtzberg presented results from clinical studies conducted at Duke University Medical Center, using autologous umbilical cord blood to treat humans with genetic and acquired brain injuries. Cord blood contains multipotent hematopoetic stem cells and progenitor cells of the immune system, and umbilical cord blood can be safely transplanted without full HLA matching. Dr. Kurtzberg has had success transplanting umbilical cord blood into children with defined genetic disorders that have neurobiological effects, including Krabbe, Hurler and Sandhoff diseases that are caused by single enzyme deficiencies. Double-blinded trails indicate that UCB transplantation can stop disease progression and might also reverse some neurological symptoms in this class of inherited disorders.

Dr. Kurtzberg and colleagues at Duke are now heading two clinical studies that target CP patients. Charles Cotten is conducting a trial to assess whether umbilical cord blood transplants improve outcomes for infants following neonatal hypoxic-ischemic encephalopathy, while Dr. Kurtzberg is administering UCB for children already suffering the effects of CP (respectively, trials NCT00593242 and NCT01147653, www.clinicaltrials.gov). These trials are the first to assess the potential therapeutic role of UCB to affect CP outcomes. First results from these trials are expected in 2013. The biological rationale for these studies is not clearly evident. One possibility is that UCB may have anti-inflammatory effects, however, complementary studies in pre-clinical animals models are needed to address the biological mechanisms of UCB stem cells..

It is clear from the clinical research discussed above that CP is extremely complex. Both injury and repair processes are protracted and occur within a critical period of neurodevelopment. Furthermore, multiple pathways and processes are activated following injury - including oxidative stress, excitotoxicity, reactive gliosis and inflammation - which lead to permanent oligodendroglial and neuronal insults. Strategies for therapeutic intervention must be similarly multifaceted if the goal of improving long-term motor and cognitive outcomes is to be achieved, and it is anticipated that these strategies will likely vary according to the nature of the injury as well as the developmental timing of the injury event.

CONCLUSION

Cerebral palsy is a disease of varied etiology and symptoms that results from brain injury during a critical time in prenatal brain development. Researchers have discovered correlations between the timing of the injury event, the location of brain

injury and the observed clinical symptoms. In the preterm newborn, the injury predominantly involves the damage and consequent dysfunction of one cell type, the oligodendrocyte (OL), during a critical period of neural development. Other cell types recruited to the sites of injury often have both injury- and repair-promoting effects on OLs. In the term infant, injury is primarily neuronal with subsequent loss of connectivity in regions that project to and from the site of neuronal loss.Although much is known about the clinical characteristics of CP, more research is required to better define the clinical parameters of the disorders and to understand how to treat this condition in a clinical setting. Specifically, there is a need to establish biomarkers and other criteria that can be used both to categorize disease progression and evaluate clinical interventions.

CHAPTER 3. UNDERLYING DISEASE MECHANISMS AND DISCOVERY RESEARCH IN CP

Research over the last 100 years has uncovered some of the most fundamental processes underlying the formation and function of the brain. In the late 19th century, Santiago Ramon y Cajal founded the field of neuroscience by showing that the nervous system consists of billions of individual cells, and developed the theory that individual neurons communicate with each other through synapses (*The Neuron Theory*). Ramon y Cajal's methods became the foundation for subsequent studies in neuroanatomy and neurophysiology in the 1930s and 1940s, which revealed the basic properties of synapses and the mechanisms underlying neurotransmission. Since then, the field of neuroscience has significantly advanced our understanding of neural development and function using a combination of animal models, *in vitro* studies of individual cell types, and improved diagnostic and imaging technologies that can be used both in animals and human. These studies complement clinical studies of human diseases. As discussed in this chapter, CP is among the many neurobiological diseases with several established preclinical animal and *in vitro* models. Modeling human diseases using a combination of animal and *in vitro* systems remains the gold standard for studying the brain, and finding treatments that can help humans using these basic approaches is the foundation of the field of translational research.

The pathogenesis of neonatal brain injury leading to cerebral palsy is highly complex. The primary injury event activates multiple overlapping responses, including cell death and proliferation, inflammation, and gliogenesis (Rezaie and Dean, 2002; Alvarez-Diaz et al., 2007). As such, CP may be considered in the context of other neuronal and demyelinating diseases such as stroke and multiple sclerosis (MS), for which there is similarly poor mechanistic insight into the molecular and cellular contributors to disease pathogenesis (Franklin and ffrench-Constant, 2008).

A. Overview of Brain Development

The adult brain consists of a web of millions of interconnected cells. The major classes of cells, neurons and glia, are generated from a common neural progenitor throughout late fetal development. Arnold Kriegstein presented a lucid overview of our current understanding of brain development in humans, based mostly on research in rodents and other animals. Neuro- and gliogenesis begins at about embryonic day (E) 9-10 in mice, when progressive waves of neurogenesis initiate within the caudal spinal cord and proceed rostrally (Rowitch and Kriegstein, 2010). The earliest progenitor of neurons and glia is the radial glial cell, which derives from the embryonic neuroepithelium and continues to proliferate within the forebrain ventricles and spinal canal as encephalization progresses. Radial glia are critical for the establishment of all major structures of the brain. In the early stages of development, they perform asymmetric cell divisions to generate neurons and intermediate progenitor cells. Following the birth of neurons, radial glia have a critical structural function by acting as guideposts for the migration of these newborn neurons to their correct cortical and subcortical layers. This process is aided by a diverse set of morphogens present within the extracellular milieu, which encourage the selective migration and differentiation of neurons into distinct neuronal cell types through a network of concentration gradients (Rowitch, 2004; Rowitch and Kriegstein, 2010; Hansen et al., 2011; Lui et al., 2011).

Once neurons have been generated and begin migrating to their cortical layers, radial glia continue to divide asymmetrically, now producing intermediate progenitor as well as

oligodendrocyte precursor cells (OPCs) (Rowitch, 2004). In the brain, OPCs are born in the ventral telencephalon and then migrate to all regions of the brain in response to cell-intrinsic as well as spatially restricted extrinsic factors (Rowitch, 2004). OPCs can proliferate to generate new OPCs throughout their migration, but once they reach a target neuron they differentiate into mature oligodendrocytes (OLs) that can ensheath contacting axons in multiple layers of myelin (Fancy et al., 2011a).

Astrocytes are essential for regulating the extracellular environment, establishing the blood-brain barrier, and supporting neuronal activity (Alvarez-Diaz et al., 2007). They are also derived from radial glia, but how and when they differentiate with respect to OPCs remains unclear. Although some *in vitro* studies have suggested the existence of a bipotent astrocyte-oligodendrocyte precursor cell, *in vivo* studies do not support the widespread existence of such a cell type, and thus the specification and lineage progression of astrocytes is incompletely understood (Rowitch and Kriegstein, 2010). Even less is known about the developmental biology of microglia, the immune scavenger cells of the CNS, although it is thought that they derive from a hematopoietic rather than a neural progenitor lineage (Alvarez-Diaz et al., 2007; Verney et al., 2010).

Dr. Kriegstein indicated that there are important differences between humans and many of the commonly used animal models. Most notably, many of the current animal systems lack gyri and sulci, which are respectively the ridges and grooves characteristic of large mammalian brains. Most animals also have markedly thinner white matter tracts as well as a much smaller proliferative zone for the production of neural progenitors (Silbereis et al., 2010; Lui et al., 2011). He also presented the discovery made in his own lab of a new type of radial glial cell, the outer radial glial cell, which is found in very high numbers in the human outer subventricular zone and can generate neural progenitor cells through proliferative and self-renewing asymmetric divisions (Hansen et al., 2010). This newly-identified cell type exists in very low numbers in rodents, but it is not clear at this time how big of a role it plays in rodent neurogenesis. This exciting finding not only identifies a new source of neural stem cells in the human brain, but might also begin to explain some of the cellular differences between rodent and human brains.

B. Animal models of CP

Throughout the CP workshop, several exciting animal models were described that have each contributed important and complementary insight into the mechanisms of white matter injury (WMI) caused by hypoxic-ischemic events. Alistair Gunn has pioneered studies in this field using a model that generated much enthusiasm among researchers; chronically instrumented fetal sheep. To induce hypoxia-ischemia, a reversible silicone occluder is placed around the umbilical cord of a gestating sheep, causing cerebral ischemia and subsequent white and grey matter damage (Dean et al., 2006; Bennet et al., 2007a; Bennet et al., 2007b). During the occlusion surgery, the fetus is also fitted with several other devices for measurement of multiple physiological parameters. These include catheters into several major arteries and veins for recordings of blood pressure, blood sampling, and drug infusion, electroencephalogram (EEG) electrodes placed over the dura to monitor electrical activity, catheters placed in the sagittal sinus to measure oxygen content, as well as several probes for the measurement of fetal cortical temperature and cortical impedance (a measure of cytotoxic edema) (Dean et al., 2006; Bennet et al., 2007a; Bennet et al., 2007b). Using this model, significant insights have been gained regarding changes in brain excitability and mitochondrial function following hypoxic-ischemic encephalopathy (HIE), as well as the timing of cytotoxic events (Hunter et al.,

2003; Bennet et al., 2007b). In addition, Dr. Gunn discovered that adenosine receptor activation, hypothermia, and blockade of electrical transmission via gap junctions suppressed the increase in electrical excitability that leads to seizure and subsequent neuronal damage (Hunter et al., 2003; Bennet et al., 2007a; Bennet et al., 2007b; O'Carroll et al., 2008). The fetal sheep was widely viewed as an excellent system for modeling human neonatal brain injury, given its long gestation period, large and complex physiology, and highly developed brain with well-formed gyri and sulci and large white matter tracts. Furthermore, given the precise experimental control allowed during gestation, including drug administration tools and physiological monitoring, the fetal sheep model was considered a highly promising system in which to study the therapeutic value of stem cells.

A smaller mammal that has also shown promise for studying the role of stem cell therapies in neonatal brain injury is the rabbit. Siddartha Tan presented a rabbit model of antenatal hypoxia at 70% gestation that results in newborn kits with early behavioral signs of CP (Derrick et al., 2004; Buser et al., 2010). Similar to humans with CP, newborn rabbits have immature locomotor function and hypertonia as well as incomplete myelination of the cerebral hemispheres (Derrick et al., 2004; Drobyshevsky et al., 2007). Dr. Tan discussed his findings regarding the therapeutic utility of tetrahydropterin and neuronal nitric oxide synthase inhibitors, and also described his current research into the potential therapeutic role of injected stem cells (Vásquez-Vivar et al., 2009). Given that the rabbit can recapitulate some of the hypertonic motor deficits observed in human CP, this model holds promise for probing whether stem cells can restore normal motor development by repairing oligodendroglial and neuronal damage.

Steve Levinson, David Pleasure, Vittorio Gallo and other researchers presented data obtained using rodent models, which are among the most genetically sophisticated model systems for the study of neonatal hypoxic-ischemic injury. Researchers studying mice, rats, and ferrets have made critical advances in understanding the basic mechanisms of disease pathogenesis, the role(s) of individual cell types in disease progression, and the identity of factors released into the injury site and their likely cell signaling pathways. Studies in these animals have also identified several potential pharmacological therapies, although none of these are currently used clinically (Tahraoui et al., 2001; Dommergues et al., 2003; Manabat et al., 2003; Fancy et al., 2009; Covey et al., 2011; Fancy et al., 2011b; Liu et al., 2011; Schmitz et al., 2011). Although rodent models cannot recapitulate the behavioral deficits associated with human CP and lack many of the neuroanatomical regions that are often damaged in humans, they offer enormous experimental advantages. These include ease of access to experimental material, high degree of experimental control, and extensive tools for probing cellular mechanisms (selective antibodies, transgenic animals, etc.). The genetic tools available make rodent models especially invaluable, allowing for the study of candidate genes by manipulating the timing and levels of protein expression. Furthermore, the rodent is already being used to study the therapeutic value of stem cells following hypoxic-ischemic insults (Chen et al., 2010; Obenaus et al., 2010). Thus, owing to their versatility and ease of genetic manipulation, rodents will remain valuable model systems for early studies of neonatal brain injury and the role of stem cell therapies.

Several promising models have been described here that each offer unique advantages for the study of neonatal brain injury. However, it was widely accepted that to apply these findings to humans, the preclinical research field must expand into animal models that more closely resemble human neurodevelopment and physiology.

For this reason, Workshop participants were strongly encouraged by the work of Sandra Juul, who has used a baboon large animal preclinical model of CP (Jacobson Misbe et al., 2011). In this model neonatal injury is induced 1-8 days before term due to asphyxia by umbilical cord clamping. Newborn reflexes, muscle tone, behavioral state, and neurological responses in newborns are all assessed according to the Brazelton Neonatal Behavioral Assessment Scale, a standard clinical method used to track human infants between the ages of 0-2 months. Assessments of motor dysfunction are carried out by physical therapists, and are accompanied by studies of early cognition, including testing for deficits in visual acuity, visual recognition memory, and social and motor behavior. At around 4.5 months the animals are tested according to the Wisconsin General Test Apparatus, a standard series of learning and memory tasks. Finally, each animal undergoes a full MRI analysis at both early (postnatal day 1) and the late (6-9 months) stages of development. These comprehensive and rigorous assessments of development are highly relevant to the human progression of this disease and reveal the power of this preclinical model.

Dr. Juul found that, following cord occlusion, animals suffered many of the developmental, neurobiological, and behavioral deficits seen in humans with CP. These include decreased Apgar scores following birth, neuronal hyperexcitability, early and sustained changes in white matter tracts, decreased neuronal connectivity, defects in muscle tone, and delays in mastering several developmental milestones compared to controls (Jacobson Misbe et al., 2011). In addition, Dr. Juul found significant changes in the concentrations of several brain metabolites, resulting in global metabolic acidosis. This last finding drew interest at the Workshop, as it could lead to the development of selective biomarkers for this disease. In conclusion, Dr. Juul's preclinical animal model represents a powerful translational system for the study of CP, not least because any therapies investigated in this context (from erythropoietin to stem cells) are more easily transferable to the clinical setting.

Although no single animal model is suitable for all of the studies that need to be done to understand and develop therapeutic paradigms for CP, it was clear from the presentations and discussions that each offered a unique set of advantages, and collectively, they have contributed to significant advances into our understanding of neonatal brain injury. As described by Zena Vexler, MRI and related imaging techniques constitute important short-term outcome measures. In order to move forward, many of the researchers agreed that future research should focus on the development of early biomarkers of hypoxic-ischemic injury, and how to incorporate stem cell therapies into animal models as preclinical evidence of safety and efficacy.

C. *In vitro* studies using animal and human stem cells

Cerebral palsy is thought to be mainly a disease of white matter injury (Silbereis et al., 2010). Like other demyelinating diseases, a major challenge in treating CP is the fact that the endogenous capacity for remyelination is severely limited, and treatments to encourage this process are lacking. Myelinating OLs are extremely sensitive to the inflammatory factors and free radicals released within an injury site. Following demyelination, new OPCs do migrate to and proliferate at the injury site; however, they fall short of differentiating into OLs and rewrapping exposed axons. In turn, this causes axonal degeneration and the collective damage that underlies CP.

In addition to the powerful animal models developed for studying neonatal WMI, a complementary and critical avenue of research is to probe the cell-intrinsic properties of the cell types involved in the injury in an *in vitro* setting. By studying the development of neurons and glia in a

controlled environment, one can examine the characteristics of each individual cell type away from the complicated injury environment. Furthermore, studies of cell-intrinsic development, reprogramming of OPCs, myelination, and even the therapeutic potential of stem cells can be carried out with tremendous experimental control. Importantly, *in vitro* preparations hold great promise as platforms for studying human disease mechanisms given that any cell type, including human cells, can be used.

Harley Kornblum provided an excellent overview of the advantages and caveats of *in vitro* studies. He cited his laboratory's extensive experience studying neural stem cells (NSCs), in both adherent cultures and neurospheres (nonadherent spherical clusters). NSCs can be taken from anywhere in the mammalian CNS. They can be isolated at virtually any time between the end of neural tube closure to the end of life, they are self-renewing when exposed to certain growth factors, and given the right environmental conditions, they can terminally differentiate into neurons, astrocytes, or oligodendrocytes. NSCs have contributed to our understanding of cell type differentiation, and could be extremely valuable for elucidating the conditions that promote oligodendrocyte differentiation into myelin-producing OLs. Furthermore, NSC-derived OPCs could be used to address how hypoxic-ischemic conditions affect their terminal differentiation. In spite of these advantages, Dr. Kornblum cautioned that any studies using NSCs *in vitro* must be approached carefully. NSCs vary in their properties depending on when (during development) and from where (in the CNS) they were isolated, and they have a high spontaneous differentiation capacity if environmental factors are not carefully controlled. Still, he argued that carefully designed experiments using NSCs *in vitro* can inform *in vivo* biology and support the rational design of *in vivo* studies.

The stem cell field has been revolutionized by the development of methods to generate human embryonic stem cells (ESCs)(Thomson et al., 1998; Chen et al., 2003; Revazova et al., 2007; Chin et al., 2010) and induced pluripotent stem cells (iPSCs) (Ohnuki et al., 2007; Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008a) that can be directed to become, among other cells, neural stem cells (NSCs). Most recently, direct reprogramming has been used to convert fibroblasts into functional neurons and cardiac cells (Ieda et al., 2010; Vierbuchen et al., 2010; Efe et al., 2011; Kim et al., 2011; Pang et al., 2011; Pfisterer et al., 2011). As described in a previous CIRM report (SCNT report), each of these stem cells offers unique advantages for generating human cells for *in vitro* studies. ESCs derived from *in vitro* fertilized embryos have been studied for decades, and serve as the gold standard for pluripotent stem cells. The ability of a single mouse ESC to give rise to a normal mouse proves that these cells are pluripotent and produce functional cells (Kubiak and Tarkowski, 1985; Nagy et al., 1990; Nagy et al., 1993; Tam and Rossant, 2003). However, the generation of ESCs is time- and resource-intensive and involves the manipulation of human embryos and other ethically sensitive procedures. For these reasons only a limited number of human ESC lines exist. iPSCs are generated by factor-mediated reprogramming of somatic cells, most commonly skin fibroblasts. iPSCs can be generated from individual patients with identified genetic backgrounds or specific clinical profiles, and offer the exciting possibility of studying cell-intrinsic properties that underlie human differences or diseases. Although single iPSCs can also produce viable mice (Kang et al., 2009; Stadtfeld et al., 2010), it has not yet been established that they are functionally identical to ESCs, and there are reports that iPSCs inherit some epigenetic features of the parent somatic cell (Polo et al., 2010; Ohi et al., 2011). In addition, reprogramming methods required to generate iPSCs are still being optimized,

and the most efficient protocols at this time involve infection of cells with integrating viruses expressing teratogenic factors that might affect the biology of the progeny cells. Furthermore, iPSC reprogramming takes several weeks. Direct reprogramming is more rapid, but as it has only recently been described very limited data exists on the quality and functional biology of these cells. However, until a genetic basis for CP is established it is unclear at this time that iPSC approaches will provide clear insight into CP pathogenesis.

These techniques provide researchers access to human stem cells for *in vitro* studies of human disease. As described above, critical advances in our understanding of the conditions that support the proliferation and differentiation of embryonic progenitors allows these stem cells to be differentiated into a myriad of CNS cell types. Phillip Schwartz gave an excellent overview of the neural stem cell field, with a particular focus on the properties of human NSCs and human ES and iPSCs reprogrammed into hNSCs (Schwartz et al., 2008; Hester et al., 2009). A number of labs throughout the world are using stem cells to develop human *in vitro* models of neurobiological diseases (Dimos et al., 2008; Park et al., 2008b; Ebert et al., 2009; Lee et al., 2009; Soldner et al., 2009; Cho et al., 2011; Soldner et al., 2011).

Marina Bershetyn described a cell-based model that employs human stem cells. Dr. Bershetyn studies lissencephaly, a human genetic disorder caused by the deletion of a large portion of chromosome 17 (containing about 20-25 genes). Haploinsufficiency of these genes results in profound defects in neuronal migration, cortical layering, gyration, neurogenesis, and neuronal excitability (Hirotsune et al., 1998; Fleck et al., 2000; Wynshaw-Boris et al., 2010). To characterize cell-intrinsic phenotypes related to lissencephaly, Dr. Bershetyn has generated iPSCs from fibroblasts isolated from lissencephalic patients. She is currently embarking on a study of the neurogenesis, migration, maturation, and neurotransmission characteristics of these "lissencephalic" iPSCs. She will also use this *in vitro* system as a platform for screening drug libraries for molecules that elevate the protein levels encoded by the missing genes. Dr. Bershetyn's work is an excellent example of how *in vitro* studies of stem cells can complement *in vivo* studies to further our understanding of complex human diseases, and as platforms for the development of novel therapeutics.

Oligodendrocyte precursor cells (OPCs) are maintained throughout life and thus represent an important reservoir of cells with the capacity to self-renew, differentiate, and potentially remyelinate axons of the CNS (Franklin and ffrench-Constant, 2008; Chong and Chan, 2010). Although our understanding of the OPC-OL transition is limited, important insight has been gained by studies performed in the laboratory of Jonah Chan. He has developed an elegant *in vitro* system whereby sensory dorsal root ganglion (DRG) neurons are co-cultured with OPCs (Rosenberg et al., 2008). He found that similar to the native developmental pattern, cultured OPCs undergo a fixed period of proliferation before differentiation. In his system, OPCs proliferated for approximately 10 days *in vitro* (DIV) prior to maturation into OLs, as measured by the onset of expression of myelin basic protein (MBP). By 20 DIV, the co-cultures were heavily enriched in myelin, owing to the ensheathing of DRG processes by mature OLs (Rosenberg et al, 2008).

Dr. Chan is using this co-culture system to identify the cellular and molecular mechanisms involved in oligodendrocyte differentiation (Rosenberg et al, 2008). First, he showed that the density of OPCs is critical for triggering their differentiation into OLs. Increasing the seeding density of OPCs at 0DIV triggered differentiation and subsequent myelination more rapidly, whereas reducing the seeding density did the opposite. Next, he showed that this process did not require dynamic neuro-glial signaling, as fixed axons and even artificial

polystyrene fibers are just as likely to be myelinated as are live axons. However, spatial and geometric constraints are critical to this myelination effect, as only artificial fibers of a certain diameter were able to trigger myelination. In addition, OPCs were induced to differentiate more quickly when surrounded by a high density of OPCs or even artificial beads of similar size to OPCs, both of which presumably "crowded" the environment. Preliminary studies indicate that human OPCs differentiated from hESCs are able to myelinate both rat axons and polystyrene fibers, similar to rat OPCs. Finally, Dr. Chan is using this system to screen for small molecules that have therapeutic potential for enhancing oligodendrocyte myelination. Dr. Chan's talk inspired much discussion about how to use his findings to improve therapeutic paradigms in animal models, how to adapt this *in vitro* system to address related questions about OL damage and repair, and how to use this versatile *in vitro* model as an assay to test the regenerative capacity of human stem cells. This work exemplifies how studies of basic neurobiological development can help establish the framework for studies into mechanisms of neurobiological disease.

These *in vitro* systems clearly illustrate the versatility of cell culture paradigms to address major questions about human diseases. Importantly, the availability of human stem cells and differentiation protocols allows these questions to be conducted not only in rodent and other animal cells but also in human cells. As indicated above, there are significant differences between animal and rodent brains, and these differences could underlie the very high failure rates observed when translating findings in rodents into novel human therapeutics for neurobiological disorders (Dragunow, 2008; Dolmetsch and Geschwind, 2011). If these differences are due to cell-intrinsic differences between human and animal cells, *in vitro* studies using human cells could improve therapeutic development for brain disorders, and could lead to more reliable diagnostic tools for human diseases. *In vitro* studies of human stem cells may therefore provide a direct link to improved clinical outcomes. Furthermore, *in vitro* models can be employed in highly creative ways to address basic questions of neurobiological development that relate to CP, particularly the relationship between axons and glia during injury and repair processes.

D. Cell generation and oligodendrocyte replacement therapies

Therapeutic regulation and/or replacement of neural stem cells hold tremendous promise for the treatment of many of the most intractable CNS diseases. One the one hand, such therapies could be rationally based on an understanding of why the brain's intrinsic repair mechanisms fail. The Wnt pathway provides an example of how OL differentiation might fail in neonatal WMI and that it constitutes a therapeutic target (Fancy et al., 2011, Nature Neuroscience). Other therapies of this kind might better marshal the repair capabilities of endogenous developing brain cells. Similar strategies are being considered in MS and thus enhanced cross-disciplinary interactions between investigators in CP and MS should be encouraged. CP is an especially relevant disease for the application of these principles governing cell-based therapies, given that the condition is generally fixed and non-progressive and results from damage of mainly one cell type, the mature oligodendrocyte. Furthermore, CP involves injury to the developing brain, meaning that improved cellular regulation or alternatively cell replacement could occur during a time in which new cells are already being added to the complex network that will become the mature organ. Given that CP might require replacement of OL-lineage cells, which are being evaluated for safety and effectiveness in other existing clinical trials (Geron trial, StemCells, Inc. PMD trial; respectively #NCT01217008, #NCT01005004,

www.clinicaltrials.gov). These factors make CP an excellent candidate for developing a human stem cell therapy.

Oligodendrocyte-based replacement is the most advanced cell therapy option in the brain (Goldman et al., 2011). Significantly, culture conditions that direct pluripotent stem cells to differentiate into OL-lineage cells or myelinating OLs have been described (Nistor et al., 2005), and these cells are being extensively characterized in culture. Stephen Huhn summarized studies showing that human neural stem cells differentiate into OL-lineage cells when transplanted into the brain (Goldman et al., 2011). Finally, the first FDA-approved clinical trial of human stem cells at UCSF Benioff Children's Hospital (San Francisco, CA) (trial NCT01005004, www.clinicaltrials.gov) involves assessing the safety of transplanting these cells into white matter to encourage production of oligodendrocyte progenitor cells (OPCs) and preliminary results indicate that the safety profile is acceptable. Final results from this Phase I study will be released in 2012. Although important issues regarding disease progression, optimal cell source, and transplantation procedures need to be addressed before cell replacement therapies can be evaluated in CP, existing experiments and trials suggest that human stem cell-based replacement therapy is ripe to be evaluated as a therapeutic option for patients with CP.

Stephen Huhn highlighted some of the issues that need to be considered when evaluating a cell replacement approach, particularly one involving human stem cells in a pediatric patient population. StemCells, Inc. led a clinical trial evaluating a neural stem cell (NSC)-based cell therapy approach for Batten's disease. Although the trial was discontinued in 2009 due to problems recruiting patients, the preliminary safety trial was positive and yielded invaluable data about the migration, engraftment and site-specific differentiation profile of their NSCs in human brain (trial NCT00337636, www.clinicaltrials.gov). Significantly, they discovered that although animal models informed the disease pathology, preclinical results of cell therapy in animals were not informative for the human trial and the transplantation protocol needed to be re-optimized in humans. The most essential aspects to successful evaluation of the therapy was a pivotal trial design with clear clinical endpoints, and the ability to track cells *in vivo.*

Results of this trial are being used to inform a current trial to replace OL-lineage cells in Pelizaeus-Merzbacher disease (PMD). PMD is caused by a mutation in the gene controlling the production of proteolipid protein (PLP), which is integral to the formation of myelin. StemCells, Inc. is collaborating with UCSF Benioff Children's Hospital investigators in San Francisco, CA, to transplant human NSCs into children with PMD, is evaluating whether the cells migrate to the brain areas requiring myelination, differentiate into myelin-producing OLs, and ensheath neurons (trial NCT01005004, www.clinicaltrials.gov). Final results from this Phase I study will be released in 2012. Similar study design and endpoints would be required to evaluate a stem cell therapy trial for CP.

CONCLUSIONS

Cerebral Palsy is a disorder caused mainly by disruption of the brain in the late stages of prenatal development, or early stages of postnatal growth, and is particularly damaging to the process of axonal myelination by mature oligodendrocytes and functional connectivity of neuronal circuits. Our understanding of the pathogenesis of the injury events, the endogenous responses to injury, and potential therapeutic interventions has been greatly enhanced by basic research. Several sophisticated animal and *in vitro* models were discussed at the workshop, and all attendees agreed that this research should continue and expand in an effort to better

understand normal neurobiological development, as well as to further elucidate mechanisms of injury. Importantly, both the animal and *in vitro* systems lend themselves well to evaluating therapies for treating CP, particularly those involving stem cells. Finally, cell replacement therapies are exciting eventual options for treating CP, and CP could be a good testing ground for stem cell replacement in the brain.

CHAPTER 4: CIRM WORKSHOP SUMMARY AND RESPONSE

The central nervous system is a complex, sophisticated organ that controls the most fundamental activities in the body, including cognitive function. Disorders that affect the developing brain are devastating and common, and have a tremendous social and economic impact. Cerebral palsy alone is observed in 2 of every 1000 live births worldwide; brain disorders affect nearly one in five young people; and an estimated four million American children and adolescents suffer from a severe mental health disorder (US DHHS, 1999). In addition, many of the neurobiological disorders that affect adults might also have originated during early brain development, as the onset of symptoms occur long after the causal processes leading to neurobiological disorders have begun. Understanding the origins of brain disorders requires studies that elucidate the mechanisms of developing brain architecture, function, and chemistry. Greater knowledge of neural development in humans and of the developmental origins of all brain disorders is essential to developing new diagnostic methods and better treatments for these disorders.

Recent advances in stem cell research and regenerative medicine offer unique opportunities to understand, diagnose and treat neurobiological disorders. Firstly, research in developmental biology and endogenous stem cells is defining the cell types present in the brain and elucidating the cell-intrinsic and environmental factors that impact the generation of these cells. Secondly, new methods in somatic cell reprogramming are allowing us to generate human embryonic stem cells, induced pluripotent stem cells (iPS cells), and neural cells. Researchers are differentiating these stem cells into neurons and glia that can be used for *in vitro* modeling and cell therapies. iPS cells carrying the genetic background of individuals with genetic forms of NDCs are being used to correlate genetic, behavioral, and cellular phenotypes. Finally, there is a new generation of clinical trials aimed at delivering neural lineage cells or human stem cells into the human brain. These exciting advances make regenerative medicine an urgent research priority for neurobiologists. Nadia Badawi summarized opinions at the CIRM workshop on cerebral palsy when she indicated that developing stem-cell-based therapies for CP should be the top research priority for clinicians and scientists in the field.

In spite of this progress, there is currently no clear path to the clinic for stem cell-based therapies for CP. Workshop participants indicated that the primary reason for this was the significant gap in our understanding of the basic and clinical aspects of neural development in humans, and a lack of research focus on childhood disorders of the brain. Arnold Kriegstein and other basic scientists indicated that more research should focus on the process of neural development, particularly in the human brain. Combining research in animal models with *in vitro* studies on human-derived cells would increase the therapeutic validity of these models. Clinically, researchers need to systematically categorize the damage observed in CP using more sensitive tools, to develop more predictive disease biomarkers, and to establish clinical endpoints to evaluate the effect of interventions.

Attendees of the workshop agree that the need for further research into the pathogenesis of CP and improved treatment approaches for CP is enormous and pressing. Basic and clinical research fields are highly complementary, but each also provides unique and critical insight into this disease. Therefore, the research required extends to all areas of biomedical and translational science, from better *in vitro* models to animal models of disease to clinical diagnostics and improved therapeutic strategies. Notably, they agreed

that stem cell therapies were a promising approach for the treatment of CP and support the further investigation of these therapies in all basic to clinical paradigms.

CIRM RESPONSE TO WORKSHOP RECOMMENDATIONS

The CIRM Cerebral Palsy Workshop resulted in a strong recommendation that CIRM target funding towards research on human brain development and neurobiological disorders of childhood, as encompassed by its mission to fund stem cell research. In addition, participants indicated that activities to support a network of clinical and basic scientists targeting CP directly would be of great benefit to the field.

Cerebral Palsy is a challenging area for therapeutic development because it is heterogeneous in etiology and in symptoms, because access to human neural tissue is limited, and because very little is known about the underlying disease mechanism(s). In spite of these challenges, CIRM agrees that investment in human stem cell research into childhood brain disorders such as CP would be of tremendous impact and could have broad applications for all human brain disorders. Importantly, the disease pathology underlying CP is applicable to a wide range of neurological and neurodegenerative diseases, most significantly to trauma or multiple sclerosis. In addition, studying neurons and glia derived from human stem cells *in vitro* will help us understand universal processes such as cellular differentiation of neurons and astrocytes, the formation of synapses, and the establishment of functional intercellular relationships like myelination of neuronal processes by oligodendrocytes. Finally, animal models of CP can be used to investigate disease pathology and address some of the challenges to successful cell therapy in the brain. CIRM therefore proposes the following:

A. Funding approach: CIRM has developed a series of flexible funding programs to advance therapeutic development at every stage of the translational pipeline, from basic science to early-phase clinical trials. CIRM, through these programs can ask the CIRM Grants Working Group and its Governing Board to prioritize research projects on CP and NDCs when making funding decisions for ongoing programs. Through these programs, CIRM could support research aimed at understanding or treating the underlying pathology of neurobiological disorders of childhood, including CP. Special emphasis would be placed on studies of endogenous human stem cells in the developing brain, on *in vitro* models of human neurodevelopmental disorders, and on studies that have the potential to advance cell therapy approaches aimed at reversing injury in the developing brain. Second, CIRM will prioritize, in funding programs such as its Tools and Technologies program, specialized research models related to CP, such as animal models. CIRM's collaborative funding network, with organizations such as NIH or private foundations are also a resource for expanding research in this important area.

B. Network approach: Dan Goldowitz described the NeuroDevNet network to promote studies of gene and cell based therapies in Canada. CIRM promotes scientific networking like this and sharing of scientific research findings by supporting meetings and by other networking activities through its Conference Grants. CIRM will support leaders in the field interested in organizing scientific meetings related to CP, with the goal of promoting networking. In addition, CIRM will consider how best to support collaborative research projects in all of the targeted funding strategies discussed above.

CONCLUSION

CIRM recognizes the tremendous social impact that cerebral palsy and other neurobiological disorders of childhood (NDCs) have in our society. Stem cell research has the potential to yield groundbreaking new tools to understand and develop therapies for CP and related brain disorders. However, the research to be done is challenging and of high risk to investigators, and in many cases requires novel interdisciplinary collaborations and an increased focus on translation. CIRM proposes to prioritize NDCs to promote research in this area.

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