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NEUROPROTECTION OF THE PRETERM BRAIN

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Abstract

Despite notable advances in the care and survival of preterm infants, a significant proportion preterm neonates will have lifelong cognitive, behavioral, and motor and we are still missing robustly effective neuroprotective strategies. These therapies must target the pathophysiological mechanisms observed in contemporaneous infants and rely on modern epidemiology, imaging, and experimental models and assessment techniques. Two drugs, magnesium sulfate, and caffeine are already in use in several units, and although their targets are apnea of prematurity and myometrial contractility (respectively), they do offer improved odds of positive outcomes. Nevertheless, these drugs have limited efficacy, and NICU-to-NICU administration varies greatly. As such, there is an obvious need for additional specific neurotherapeutic strategies to further enhance the outcome of this very fragile population of neonates. The present chapter reviews these issues, highlights bottlenecks that need to be solved for meaningful progress in the field, and proposes future innovative avenues for intervention, including delayed interventions.

Key words

Encephalopathy of prematurity, abnormal brain development, dysmaturation, tertiary phase, microglia, oligodendrocyte maturation, melatonin, magnesium sulfate, EPO, stem cells, stratification

1. Pathophysiology of the encephalopathy of prematurity (EP)

It can be estimated that 9 of the 15 million infants born preterm every year (60%) will suffer from life-long physical or neurological handicap, largely due to brain injury(1-4). The hallmarks of their injury are: hypomyelination, oligodendrocyte maturation arrest, axonopathy, neuroinflammation, reduced fractional anisotropy (FA) and cortical volume determined by magnetic resonance imaging (MRI), and eventually, significant cognitive deficits(5). Collectively the brain damage associated with preterm birth is called the Encephalopathy of Prematurity (EP). Maternal-fetal infection not only precipitates preterm birth, but is often clinically silent and a major driver of EP(6). Specifically, a wealth of epidemiological and clinical studies have shown that perinatal systemic inflammation, linked to chorioamnionitis, funisitis, postnatal sepsis or necrotizing enterocolitis, is a key risk factor in developing EP and its associated long-term neurological and behavioral/psychiatric deficits, see reviews(7,8). Also, in some cases like placenta abruption or twin-twin transfusion, hypoxic-ischemic insult is a notable risk factor (9). Based on animal models of white matter damage, chronic hypoxia(10) is also considered another risk factor of EP, but conclusive supportive clinical data is still missing and the validity of this concept is contested(11). A more convincing argument is that hyperoxia may be involved in the pathophysiology of EP(12), as the *ex utero* environment is relatively high in oxygen compared to the *in utero* environment (13). That hyperoxia plays a role in EP is also supported by animal studies(14-16).

In regards to the severity of injury to the brain, the most recent postmortem neuropathology studies (17,18), supported by MRI studies (19), clearly indicate that we have moved, over the last two decades, away from typically destructive white matter lesions, the so-called cystic periventricular leukomalacia. We have moved towards non-clastic diffuse white matter abnormalities, i.e., diffuse white matter injury, on top of which can be superimposed some small focal necrotic lesions. Also, MRI studies, have shown abnormal connectivity and abnormal grey matter microstructure play an essential role in the long-term deficits (20-23).

However, these MRI studies (and all other MRI studies) rely on assumptions of the cellular substrate underpinning the imaging signal, and although some work is underway to understand this(24), more needs to be done. Recent unpublished post-mortem studies also support the loss of a specific subset of cortical interneurons that play a crucial role in neuronal plasticity during development (Stolp & Gressens, unpublished data). This observation refines previous post-mortem observations that total proliferation in the ganglionic eminence (GE) is reduced in preterm born infants suffering from intra-ventricular hemorrahage(25). The sensitivity of interneurons to disruption due to preterm birth relates to their on-going migration from the dorsal subventricular zone (SVZ) and GE (ventral SVZ) during the third trimester(26). The validity of changes in interneurons has also been pre-clinically demonstrated in a rabbit model of preterm birth(27) (27).

1.1. Neuroinflammation, and microglial activation

Microglia are the innate immune cell of the brain but during development they have critical functions in synaptic pruning and as such regulating large scale connectivity(28-30), and there are even pro-myelinogenic microglial subtypes(31,32). Microglia share a great deal of characteristics with macrophage but numerous cell fate mapping studies have established that they are a unique suptype of cell(33,34). Microglia are activated as a consequence of systemic inflammation or injury to the brain. Systemic inflammation activates microglia indirectly by acting via interleukin-1 (IL-1) receptors on the vast network of endothelial cells coating the blood vessels in the brain. IL-1 is an almost ubiquitous cytokine found in the blood during infection or injury(35). Activation of these endothelial cell situated receptors leads to the production of pro-inflammatory prostaglandins in the parenchyma that activate microglia. Microglia are also activated by brain injury, such as hypoxia, or HI, as these events lead to cell injury and the release of damage associated messenger proteins (DAMPS) and/or the production of toxic metabolites that also active microglia directly(36,37). In vitro,

hypoxia and hypoxia have been shown to themselves have a direct effect of microglia, including by altering receptor and transcription factor expression(38-40). The activation of microglia, is a general term to describe a disruption away from their typically homeostatic roles in brain development, maintenance and surveying. We characterize the activation state of microglial based on their expression of receptors, enzymes and functions such as phagocytosis, that altogether give us clues as to the overall effect of the cell on the brain, or invading pathogen. Activation can take a number of forms, and in paradigms of brain injury, we need to create simplified nomenclature, but in reality, cells can transition between activation states and express markers of multiple states. The general activation states of microglia include a M1-like classically pro-inflammatory state, M2a classically anti-inflammatory or regenerative state and M2b immune-regulatory activation profile(41). Of note, microglia activated to a pro-inflammatory state generate pro-inflammatory cytokines and reactive oxygen species (Figure 1). Numerous in vitro systems and in vivo models of EP have shown that microglial activation can lead to oligodendrocyte precursor (OPC) blockade or death and subsequent myelin deficit (please see next section) (10,42-44). In addition, activated microglia will lose their normal functions in synapse development, function, and plasticity, having potentially a detrimental effect on neuronal differentiation and connectivity. This double hit (production of toxic factors for neighboring neural cells and loss of normal microglial functions to shape axonal connectivity and synaptic elimination/function) has emerged recently as one key hallmark of EP.

1.2. Oligodendrocyte precursor cell maturation blockade and dysmyelination

The latest neuropathological studies have shown that, except in the most severe cases of EP that OPC maturation blockade rather than OPC death was the key feature of diffuse white matter injury (17,18). For excellent reviews including oligodendrocyte maturation in the context of perinatal brain injury please refer to (45,46). One has to remember that preterm infants who die are generally at the most severe end of the spectrum, and postmortem

studies are potentially biased by the local standards for clinical care and the local ethical basis for withdrawing care. As such, when studying neuropathological events with the aim of designing neurotherapies that the target is indeed the majority of preterm born infants survive the perinatal period. Similarly to the human condition, in animal models, moderate systemic inflammation leads to OPC blockade and hypomyelination (44,47). Moderate inflammation is used to model the great number of preterm born infants born suffering a sub-clinical level of infection but who are still at a higher risk of EP and poor outcomes(48,49). While more severe insult models (i.e., severe systemic inflammation, protracted hypoxia or hypoxic-ischemic insult) lead to a combination of OPC death followed by proliferation of OPCs and maturation blockade of these newly formed cells (10,44,50).

The mechanisms by which OPC maturation is blocked center on responses to the neuroinflammatory milieu, for a full review see(51). It has been demonstrated in animal models that OPC maturation blockade is caused by exposure to inflammatory prostaglandins (52), soluble factors called Wnts(53) and extracellular matrix proteins, the hyalurans, via their activation of damage associated protein receptors (54).

In all rodent models that we have seen, over time (at a time-point equivalent to young adulthood or late puberty) the deficits in OPC maturation/myelination resolve themselves for reasons in part linked to the naturally higher rate of turnover of OPC in rodents compared to humans (55). The human brain exchanges roughly 0.3% oligodendrocytes per year in adulthood, but the mouse exchanges approximately 10-30 times that number; 1-3% oligodendrocytes per year(56). However, despite this normalization of myelination levels, behavioral impairments do persist in rodent models well into adulthood. In humans, long-term MRI studies indicate that abnormalities in structure of the white and grey matter persist well into adulthood(57-59). Histological correlates of these are almost impossible to study as most preterm born infants without substantial gross motor impairments, and as such co-morbidities such as epilepsy, live to a close to normal age. As such, preterm born infants born into the current high standard of NICU care are not yet having their brains banked for any studies.

1.3. Specific deficits in interneuron subtypes

Preliminary postmortem and experimental studies suggest some specific deficits of some subsets of interneurons at least in the cortex, as outlined above. Interneurons play a key role in the balance between excitation and inhibition. In addition, some interneurons like parvalbumin interneurons are instrumental for neuronal circuit plasticity (60), and recent studies suggest that this specific subset of interneurons is decreased in the cortex of preterm infants. The underlying mechanism, possibly reduced production, abnormal migration, reduced survival, lack of differentiation, is unknown. However, in preterm born infants suffering from an intra-ventricular hemorrhage, proliferation within the region of the brain responsible for the production of interneurons, the ganglionic emminance, is reduced(25). Studies in humans are time consuming as the vast majority of the brain needs to be mapped in many infants to determine the nature of a numerical or migration change.

1.4. Impaired connectivity, neuronal differentiation, synaptogenesis

Almost all of the data available on grey matter changes in EP are from imaging studies, or from animal models. Imaging studies have focused on microstructural alterations, and have begun to apply complex models derived from developmental data and our general understanding of brain development (23,61). However, a limitation of these models is a lack of specificity between MRI signal and neuropathological substrate, as illustrated in a recent paper using a technique where the hippocampus was assessed with high field MRI and also subjected to immunohistological analysis and detailed co-registration(24). Of interest, this study found that fractional anisotropy correlated with astrocyte density a cell types typically not considered in MRI studies. Further studies will be necessary to determine the precise cellular and molecular substratum of abnormal connectivity and grey matter microstructure observed on MRI. Large animal models of severe EP, have illustrated that grey matter

deficits include changes in dendritic arborisation in subplate neurons (62) and also impaired expansion of the dendritic arbor and reduced synaptic density from the pyramidal cells of the hippocampus(63).

1.5. Tertiary phase

Many months and years after injury to the immature brain there may be persisting injury processes, in addition to developmental disruption associated with the initial brain insult (64). These tertiary mechanisms of damage might include persistent inflammation and altered epigenetics and are implicit in preventing endogenous repair and regeneration, predisposing to development of later cognitive dysfunction and sensitization to further injury (Figure 2). Tertiary mechanisms of damage might be treatable, including by preventing the repressive effects of microglial and astrocyte over activation, recapitulating developmentally permissive epigenetic conditions, and using cell therapies to stimulate repair and regeneration. Recognizing tertiary mechanisms of damage might be the first step in a complex translational task to tailor safe and effective therapies that can be applied to the already developmentally disrupted brain long after an insult. Clinical data accumulates that the tertiary phase of injury can be observed. Initially, positron emission spectroscopy demonstrated that many years after an adult TBI that the human adult brain had on-going glial reacvitiy (65). More recently, Evidence for tertiary phase proceses after PBI includes (i) abnormal cortical metabolite levels detected by MR spectroscopy (MRS) at 1 yr of age after hypoxia-ischemia at term birth(66), and in adults who were born preterm(67), and (ii) increased serum pro-inflammatory cytokines and heightened responses to endotoxin re-challenge in 7 yr old children with cerebral palsy after preterm birth(68). Consistent with these chemical changes are the clinical observations that cerebral palsy, a severe complication following perinatal brain injury, is an independent risk factor for adult stroke(69); that preterm birth is a risk factor for dementia-associated neurocognitive impairments(70) and neuropsychiatric disorders such as

depression in later life(71). Indeed, for those born preterm, health and social status deficits are greater in the 4th decade compared with the 2nd decade of life(72). Furthermore, experimental studies also show that early life exposure to inflammation can predispose to later increased brain injury severity, and even increase the severity of alzheimers disease or multiple sclerosis(73-76). The chief candidate for mediating these effects are microglia. Of note, microglia are long-lived, in the human brain this is estimated to be up to 20 years and in the mouse for their entire lives(77,78). After brain injury microglia number and function is altered for many months and even years in humans(65) and non-human primates(79), and into adulthood in experimental models of perinatal brain injury(73,80-82). Work to link the imaging data and serum biomarkers indicating tertiary phase changes with neuropathological changes that are viable neurotherapeutic targets is still underway. However, we speculate that anti-inflammatory drugs with good tolerability administered over long periods of time might be capable of incremental improvements in neurological outcomes for people suffering the consequences of EP.

2. Current therapies, recent and ongoing clinical trials

Neuroprotection of the preterm brain is a health care priority both in terms of suffering and economy. However, the commitment of drug makers within the field of perinatal neuroprotection remains scarce despite positive measures put into place by the EMA and the TGA to support paediatric drug development. What are the potential reasons for this lack of investment? i) Perinatal neuroprotection does not represent a big financial market with relatively few patients treated once for a few days or a few weeks; ii) pharmacokinetics, metabolism, interactions with other drugs, and potential side effects are poorly understood in new-borns and might change with gestational age at birth; this combined with the fact that new-borns (and especially very preterm new-borns) are fragile potentially increases the risk for adverse outcomes in clinical trials, which could lead to legal procedures and have a very

negative impact for the reputation of the drug company in the general public; iii) drugs may leave markers in development which are not necessarily due to the drug, but due to the combination of the reduced lesion and the development stage, and these markers may appear in cohorts; thus there is the possibility of adolescents suing drug makers, where their lives have been saved, but certain developmental markers remain which might be, rightly or wrongly, ascribed to the drug. For these types of reasons, the pharmaceutical industry is reluctant to engage in this area. In this context, academic laboratories, in most instances, do not have the capacity to develop new drugs for a given indication. Therefore, re-purposing existing drugs is most likely the shortest way to improve the outcome of preterm infants in the coming years.

2.1. Magnesium sulfate

There have been controversies related to the use of this magnesium sulfate, due to a conflagration of issues such as unrelated case reports and biased opinion publications. Nevertheless, numerous RCTs have tested the efficacy of magnesium sulfate given to mothers at risk to deliver prematurely throughout the world during the last decade. Although the number of included patients in some trials was not sufficient to reach significance, all trials showed the same tendency for neuroprotection. A recent meta-analysis (83) of five randomized trials, including a total of 5,493 women and 6,131 babies, unambiguously shows that antenatal magnesium sulfate given prior to preterm birth reduces motor problems (CP) and reduces the combined risk of fetal/infant death or CP. Although the effect sizes are not large they still significant, and the authors rightly state that “widespread adoption worldwide of this relatively inexpensive, easy-to-administer treatment would lead to important global health benefits for infants born preterm” (83,84). This positive endorsement for magnesium sulfate as a neuroprotectant is also found in a 2009 Cochrane review (85) and in scientific impact paper published by the Royal College of Obstetrician and Gynecologists in the

UK(86).

2.2. Caffeine

Caffeine is a xanthine that is used in centers all over the world to prevent and treat apneas of prematurity due to its action to stimulate the central nervous system and respiratory muscle function. Through its nonselective antagonist effects on adenosine receptors (AR), caffeine also has potentially multiple impacts on the developing brain of a preterm infant(87). This includes effects to induce the precocial maturation of oligodendrocytes, which if stimulated too early in development could reduce the pool of oligodendrocytes present to produce myelin in later life. In addition to AR antagonism, caffeine has other biological actions, including inhibiting phosphodiesterases (PDEs) (e.g., PDE1, PDE4, PDE5), promoting calcium release from intracellular stores, and interfering with GABA-A receptors.

Human studies have shown that caffeine significantly reduced motor deficits in preterm infants without impacting negatively on their cognitive outcome at 11 years of age (88). Increasing the doses of caffeine would be tempting to potentially further increase the neuroprotective effects. However, we should keep in mind that adenosine receptors are highly expressed in the developing brain and are playing important modulating roles(89). RCTs seem the only way to address this issue. For a discussion of the issues surrounding providing care in the NICU and off target effects of drugs (and their excipients) we point the reader to an aging, but still pertinent review(90).

2.3. Erythropoietin (EPO)

Erythropoietin *(*EPO) has been used safely for decades in preterm infants to reduce the necessity for blood transfusions. EPO also has neuroprotective effects in animal models of

term brain injury and some immature paradigms(91-94), although at higher doses as the blood-brain barrier penetrance is not optimal. A RCT with EPO given after preterm birth has been conducted in Switzerland. The MRI evaluation of brain lesions at term corrected age in a subset of patients showed a significant decrease of the lesion burden in the EPO arm (95). However, the 2-year age neurodevelopmental evaluation on the whole cohort failed to show any significant benefit (96). In parallel, a large-scale trial has been initiated, the PEANUT trial, into which 940 patients will be enrolled at 19 sites across the United States in order to evaluate 752 infants at 22–26 months corrected age (97). Although the safety of EPO is well established and the idea of re-purposing this drug as a neuroprotectant is valid, one has to stress that there is no pre-clinical data showing the neuroprotective effects of EPO in a model of EP, involving the contemporaneous constellation features of EP. This raises questions about whether we are still best placed to understand the best paradigm to delivery neuroprotection in this population.

2.4. Melatonin

Melatonin is very safe including at very high doses in preterm infants (98) and its pharmacokinetics has been established in very preterm infants (99). Melatonin has multiple protective effects including anti-oxidant, trophic, anti-apoptotic, mitochondria-protective, and anti-inflammatory effects (98,100). In addition, preterm infants are not able to produce their own melatonin and, while losing their maternal daily supply, are deprived of melatonin when compared to age-matched fetuses. Clinical trials are ongoing exploring the optimal dose and timing (pre- vs post-natal or combination of both) of exposure. However, these studies are facing the issue of formulation, specifically, that melatonin is very difficult to dissolve, especially in a small volume compatible with a tiny preterm neonate. It is currently formulated with ethanol as an excipient although companies working on improved formulation. However, we do with to highlight that many drugs used in the NICU are dissolved in ethanol, and other

compounds considered toxic to the developing brain(101-103). Perhaps melatonin in its current form is a viable neurotherapeutic, if we were better able to stratify patients at the highest risk of EP and long term functional injury and for whom the risk-benefit ratio was sufficiently well balanced (see, Stratification section belwo).

2.5. Breast milk

Neuroprotection can be based on therapies that need to be FDA and EMA approved, such as magnesium sulfate, on limiting toxic environment, for example choosing the right steroid for lungs, such as hydrocortisone vs dexamethasone, or by changing our non-treatment realted practices. In this regard, “predominant breast milk feeding” in the first 28 days of life was associated with a greater deep nuclear gray matter volume at term equivalent age and better IQ, academic achievement, working memory, and motor function at 7 years of age in very preterm infants” (104), demonstrating that we have the ability to implement a therapy, with no side effect at all, that is a key step forward protecting the brain of preterm infants. Difficulties in implementing breast milk feeding in the NICU include maternal poor health and stress due to the preterm delivery, social factors related to the lower than optimal rates of breastfeeding the general community, and linked to this poor access to lactation support within the hospital. Milk banks providing donated milk to infants has had some success, and although these endeavors are expensive they are likely no more so than poor infant outcomes, or novel therapy design.

3. Future directions

3.1. Early vs delayed intervention and need for stratifications through biomarkers

Obviously, the earlier one could intervene in a pathological process, the more chance to block or slow down it and prevent subsequent long-term consequences. At the beginning of the 21st century, this universal truism still faces major limitations in the field of neuroprotection of preterm neonates. First, very and extremely preterm neonates are extremely fragile facing the extreme immaturity of all their organs making the encounter with the ex-utero and NICU environment very challenging. Any additional therapeutic intervention is always a risk factor to further jeopardize their precarious homeostatic situation. Second, within the population of very and extremely preterm neonates, despite the risk of long term consequences, a significant number of them will do well. These two facts have major implications in terms of neuroprotection of preterm infants. Two complementary avenues of research could help to resolve this low sensitivity/specificity of current prediction paradigms. One would be to delay interventions (based on the tertiary phase concept that opens up a much larger window for intervention) and only treat infants with clearly identified abnormalities or major risk factors (such as signs on sophisticated MRI) of adverse outcome. In this context, one has to keep in mind that for some neurodevelopmental processes, timing is particularly important. For example, restoring myelination at a distant step from normal timing might not be sufficient to restore the function. The alternative approach would be to identify very early (first hours/days of life) sensitive and specific biomarkers of negative outcome. These latter could include a combination of blood/urine-derived markers based on Omics studies on circulating blood cells (i.e. micro-RNAs, mRNA, proteins, metabolic markers) and SNPs, imaging being performed at bedside such as ultrafast Doppler and related techniques. (105).

In the hunt for biomarkers for outcome recently, an integrative analysis of data from an animal model of EP together with human genomics data (SNPs associated with white matter abnormalities on MRI) has allowed the identification of potential molecular targets relevant to preterm infant brain injury and also predisposing infants to poorer outcomes(106,107). Genomics-imaging studies of this type have identified SNPs in fatty acid metabolism(108),

the transcriptional regulator PPARg(107,109), and in Wnt pathway(110), which in the future may provide us the ability to screen for specific genotypes as risk stratification guides.

3.2. Improving outcome measurements

The efficacy and importantly the true safely of a strategy can only be judged over a long period of time, and definite outcome of interventions in preterm infants require at least 6-10 years for every clinical trial. If the rate of attrition of clinical trials for preterm birth is comparable to a disease like Alzheimer disease, several decades at least might be necessary, unless luck is on our side, to uncover a new additive protective strategy. Although long-term RCT will remain the gold standard, trials based on early proxies, like for example sophisticated MRI at term corrected age on smaller samples might serve as a "triage" pipeline to select the best candidates for future classical RCTs. As an example of this approach we would like to highlight a study conducted by the team at the Center for the Developing brain at King's College London (KCL). They validated across cohorts of term born infants diagnosed with encephalopathy related to hypoxia-ischemia (HIE) that a positive outcome could be reasonably predicted by a reduction in lactate to N-acetyl aspartate ratio in the thalamus and in preserved fractional anisotropy in the posterior limb of the internal capsule (PLIC), measured with magnetic resonance spectroscopy and MRI, respectively, measured within 15 days of birth (111).Using these short outcome measures they were able to disprove the hypothesis that inhaled Xenon would be a viable adjunct therapy for hypothermia which is the standard of care for term born infants with HIE, but that has a limited efficacy. The team from the Centre for the Developing Brain at KCL have tested the sensitivity of Tract-Based Spatial Statistics (TBSS) to a simulated treatment effect in preterm neonates successfully, but this has not yet been applied to a patient cohort (22) As such, currently the first robust outcome data is available only at 2 years of age for preterm born infants. Imaging modalities have become better at measuring micro-structural abnormalities

(112,113) despite our limited understanding of the specific cellular nature of the signals(24) and that once a clinical trial is put into place in this center that the usefulness of TBSS as a proxy can be properly tested.

3.3. Manipulating the inflammatory milieu

As described above microglia play a substantial role in the neuroinflammatory processes leading to EP and these cells can display phenotypes with diverse functions including pro-inflammatory or toxic, anti-inflammatory or immunomodulatory, pro-repair or pro-plasticity, plus other emerging phenotypes(32,114). In this context, blocking the activity of all microglia can be deleterious as shown in models of term infant HI injury (115) and also adult HI injury(116). The difficulty in pan-supression of inflammation can also be illustrated in the history of minocycline treatment for the disease ALS, amyloid lateral sclerosis. Although inflammation is considered central to the neuropathology of this disease treatment with the broad anti-inflammatory minocycline excascerbated patient symptoms (117).

Astrocytes also play a key role in neuroinflammatory reactions, although their specific role has not been heavily studied in preterm infant post-mortem tissues. It has been shown previously that there is a gestational age dependent activation of astrocytes associated with white matter injury, where in very preterm born infant brain (i.e., 24-29 weeks gestational age) there is no astrogliosis, but astrogliosis is present in infants classified as preterm (i.e., 30-34 weeks gestational age)(118). Astrocytes have also been classified into the subtypes, the A1 neurotoxic and A2 regenerative subtypes based on their expression of receptors, cytokines, chemokines and enzymes(119-121). Although these phenotypes are less studied than microglia and in particular how they relate to the developing brain is poorly understood. Currently little is known about how to target astrocytes specifically to reduce EP, but experimental evidence suggests a cross talk between microglia and A2-associated astrocyte mediated by the prostaglandin COX2 is involved in EP(52). Targeting COX2 production

globally with nimesulide in this paradigm proved to be neuroprotective against EP. However, the nimesulide treatment of non-injured mice in this model part of a set of controls, caused hypermyelination, highlighting that processes generally classified as inflammatory have in normal brain development and towards the need for robust treatment criteria.

A recent study has provided for the first time in a large animal model of the preterm infant exposed to an endotoxin (i.e. chorioamnionitis), data on the effect on the brain and systemic inflammatory markers over a long period – a total of 8 days. It is possible to identify two waves of changes in response to endotoxin exposure, including in systemic inflammatory mediators and neuronal architecture(51). As such, without clear biomarkers for the stage and severity of inflammation (including the states of microglia and astroglial activation) we are still unable to design neurotherapeutic trials that optimize the chance of success.

3.4. Oxidative stress

Although targeting reactive oxygen stress has been a failure in most clinical trials for a large number of disorders, the use of safe anti-oxidants in combination with other drugs targeting other cell processes might have and added value. Allopurinol, N-acetyl-cysteine, curcumin, and other molecules could belong to this category (122,123). We believe that increasing information about the necessary role of classically pro-inflammatory microglia to lessen injury volumes, perhaps by setting up the repair and regeneration phases (as outlined above) in the acute phase of injury explains the failures of anti-ROS therapies.

3.5. OPC maturation

Acting directly on OPC to promote their maturation is an obvious strategy. Basic neuroscience has taught us a lot on the molecular mechanisms controlling the different steps of maturation of OPCs to reach a fully mature oligodendrocyte stage that is able to produce myelin. In addition, the field of multiple sclerosis (MS) is facing a somewhat similar issue with some newly formed OPCs failing to mature and to repair the demyelinating lesion. Based on the fact that preterm infants are somewhat deprived of thyroid hormones that are important for OPC maturation, a supplementation of T3/T4 has been suggested. However, animal models have shown a failure of T3/T4 to improve the myelin status (124,125). Targets identified in MS animal models and currently tested in clinical trials include histamine-3 receptor(126) that was recently shown to be neuroprotective as well in a model of EP (Gressens and Chuang, unpublished data).

3.6. Connectivity and interneurons

As mentioned above, despite the fact that grey matter abnormalities might play a very important role in the long-term consequences of prematurity, there is not currently enough knowledge to make any specific hypotheses on the potential targets for neuroprotection. Generally, these are likely to focus on cell specific delivery of drugs that can reactivate earlier developmental programs to support integration steps that may be been disrupted, especially for interneuons. Also, more speculatively, we know that microglia are responsible for manipulating the connectome throughout life (28,29,127) and we know that microglial functions remain altered for many months if not years after brain injury(64,65). Taking these two facts together, we can assume that in the future, cell specific manipulation of microglial functions will give us the ability to enhance the beneficial effects of physical and cognitive training on the connectome in people with brain injury.

3.7. Stem cells and microvesicles

In term infants with neonatal encephalopathy, mesenchymal stem cells (MSCs) appear at this stage as the most promising stem cells for therapy. MSCs have anti-inflammatory and pro-repair effects and stimulate the production and survival of endogenous stem cells (128). In addition, in rodents and non-human primates they can reach the damaged brain when administered intra-nasally. Of note, at least in cell culture, they release a significant amount of microvesicles that contain numerous types of biological materials (lipids, enzymes, mRNA, micro-RNAs, …) that can cross-talk with other cell types and that might mediate some of the positive effects of MSCs (129). The safety of MScs in preterm infants is totally unknown. In this context, after having solved the issues of timing and single vs repeated administrations, route of administration, doses, and optimal type of MSCs, the safest place to start might be in a delayed manner in infants where abnormalities have been confirmed.

3.8. Nanoparticles and cell-specific targeting

Especially during normal brain development, different cells types use similar pathways, sometimes with antithetical effects (for example, OPCs shut down Wnt pathway to mature while Wnt pathway shut down in microglia promotes their toxicity). Therefore, being able to target specifically one cell type could increase efficacy while limiting side effects. Engineered nanoparticles used as carrier for therapeutics might help to improve cell specificity (123). Evidently, efficacy and safety (in particular lack of long-term accumulation in the brain and organism) of these nanoparticles has to be validated in the first place.

3.9 Harnessing the power of pretreatment

Two recent studies have provided a possibly revolutionary tool to the field of preterm infant care; a non-invasive test that can predict preterm delivery. In the first study of 400

women(130), a blood test taken at 15 to 20 weeks gestation was able to predict 80.3% of women who went on to have any preterm birth using a matrix of values from 25 variables in the blood. Importantly, it had a 95% predictive ability for women who would give birth before 32 weeks due to pre-eclampsia. The second smaller study undertook to develop a test for measuring gestational age and for predicting preterm birth(131). Firstly, they found that with a combination of 7 cell free RNAs from the blood they could predict gestational age with the same accuracy as a first trimester ultrasound. Second, also using a battery of cell free RNA markers they were able to predict preterm birth in a high risk cohort more than 2 months before delivery. It is worth noting that both of these studies will require extensive cross-cohort validation, but they are exciting advances in our field.

Although for some time now, in high risk women, cervical length and cervical fetal fibronectin length have had strong predictive values for imminent delivery(132). However this testing is not effective for low risk women(133). In addition, multi-modal screening tools have been designed that have excellent specificity and sensitivity, but these also rely on multiple invasive procedures and have lesser efficacy in low risk populations(134). The importance of identifying women who will go on to deliver preterm cannot be understated as preventing rather than treating an injury is always the preferred approach. It's importance is underscored by the fact that a small study has shown using fetal MRI, that functional connectivity in the fetal brain is already decreased before a pre-term delivery(135). The authors state that 'first human data to suggest that disabilities frequently accompanying extreme prematurity, such as autism and ADHD, may derive from pre-existing intrauterine neurological conditions, especially given that these disorders have neuroconnectional bases'. Together with an ability to determine which babies will be born early, in depth fetal imaging will undoubtedly provide more information regarding what the initial triggers for preterm birth are and as such, avenues for novel therapies.

4. Conclusion

Despite significant improvement of survival and outcome over the last decades, a significant proportion premature infants have long-term cognitive and behavioral deficits. The search for efficient and safe neuroprotective strategies for this high-risk population remains a heath care priority. Novel targets and candidate drugs have been proposed. To maximize the chance of conclusive RCTs, it seems necessary to have solid preclinical data, a good design of the RCT with stratification of infants based on early biomarkers, and sensitive outcome measurements.

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6. Legends of Figures

Figure 1. Schematic representation of the pathophysiology of EP induced by inflammation.

Figure 2. Outline of the acute, secondary, and tertiary damage phases in the EP (adapted from (64))

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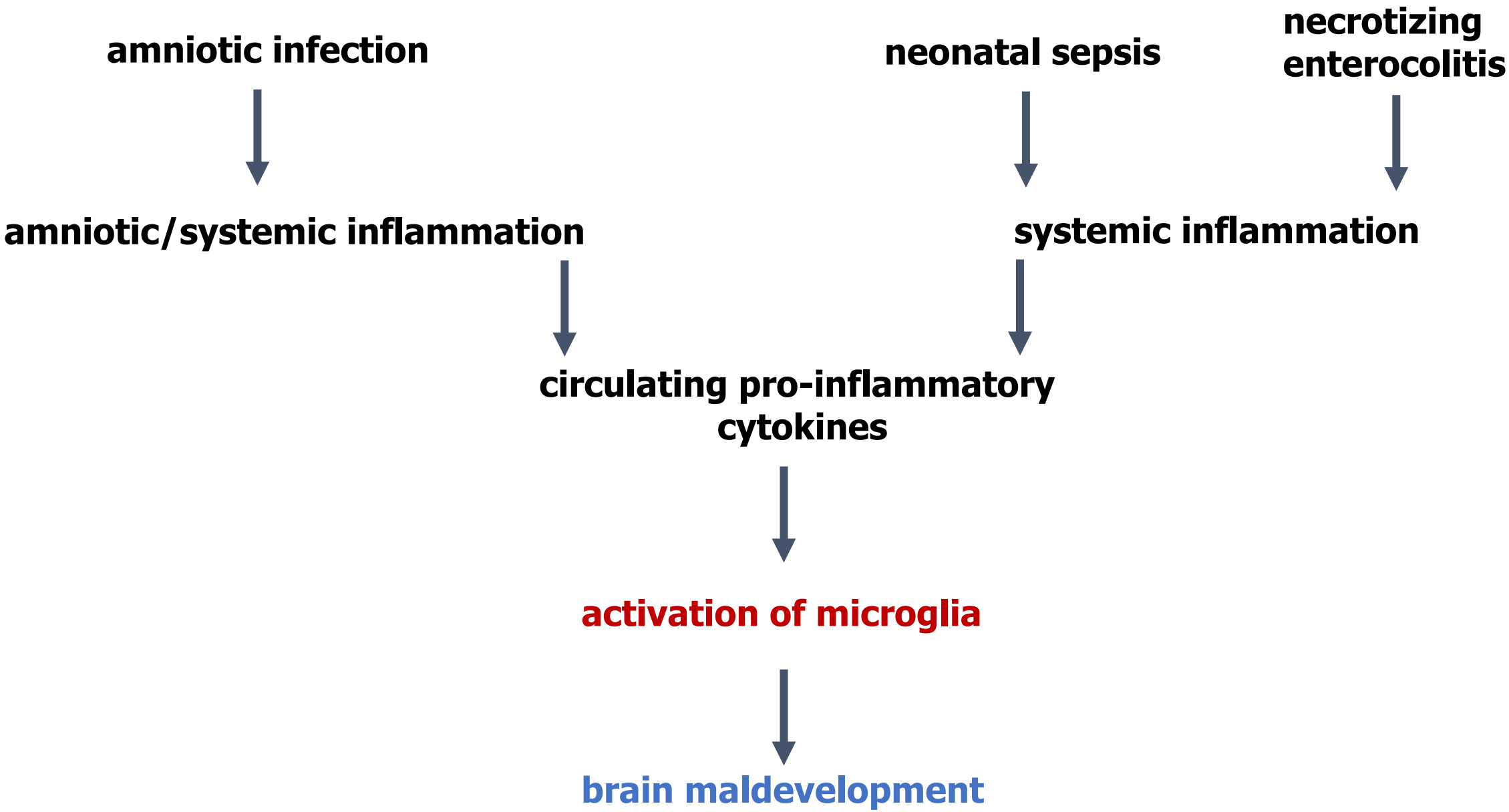
amniotic infection
neonatal sepsis
necrotizing enterocolitis
amniotic/systemic inflammation
systemic inflammation
circulating pro-inflammatory cytokines
activation of microglia
brain maldevelopment

Fig 1

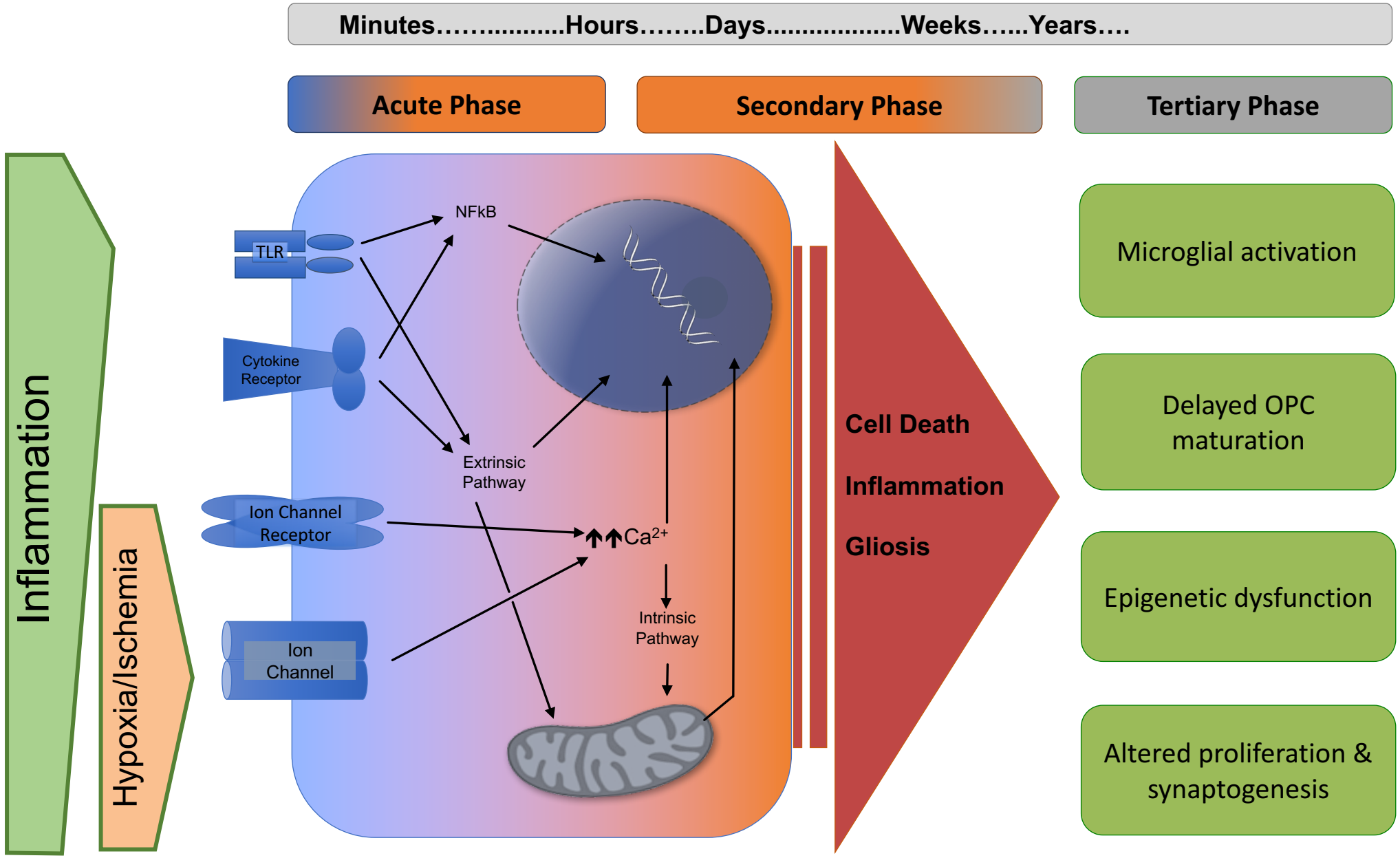
Minutes……..Hours……..Days……..Weeks……Years….
Acute Phase
Secondary Phase
Tertiary Phase
Inflammation
Hypoxia/Ischemia
TLR
Cytokine Receptor
Ion Channel Receptor
Ion Channel
NFkB
Extrinsic Pathway
Intrinsic Pathway
↑↑Ca2+
Cell Death
Inflammation
Gliosis
Microglial activation
Delayed OPC maturation
Epigenetic dysfunction
Altered proliferation & synaptogenesis

Fig 2