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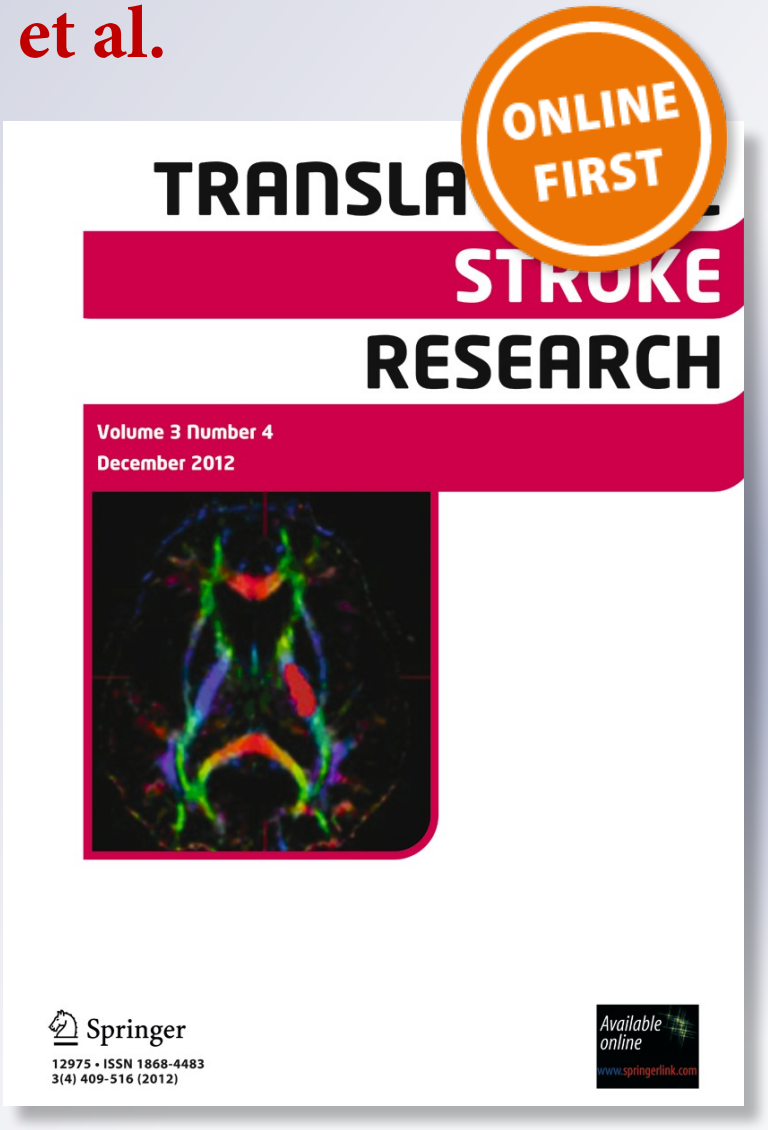

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REVIEW ARTICLE

Bench to Cribside: the Path for Developing a Neuroprotectant

Nelina Ramanantsoa · Bobbi Fleiss · Myriam Bouslama · Boris Matrot · Leslie Schwendimann · Charles Cohen-Salmon · Pierre Gressens · Jorge Gallego

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Abstract The consequences of perinatal brain injury include immeasurable anguish for families and substantial ongoing costs for care and support of effected children. Factors associated with perinatal brain injury in the preterm infant include inflammation and infection, and with increasing gestational age, a higher proportion is related to hypoxic–ischemic events, such as stroke and placental abruption. Over the past decade, we have acquired new insights in the mechanisms underpinning injury and many new tools to monitor outcome in perinatal brain injury in our experimental models. By embracing these new technologies, we can expedite the screening of novel therapies. This is critical as despite enormous efforts of the research community, hypothermia is the only viable neurotherapeutic, and this procedure is limited to term birth and postcardiac arrest hypoxic–ischemic events. Importantly, experimental and preliminary data in humans also indicate a considerable therapeutic potential for melatonin against perinatal brain injury. However, even if this suggested potential is proven, the complexity of the human condition means we are likely to need additional neuroprotective and regenerative strategies. Thus, within this review, we will outline what we consider the key stages of preclinical testing and development for a neuroprotectant or regenerative neurotherapy for perinatal brain injury. We will also highlight examples of novel small animal physiological and behavioral testing that gives small animal preclinical models greater clinical relevance. We hope these new tools and an integrated bench to cribside strategic plan will facilitate the fulfillment of our overarching goal, improving the long-term brain health and quality of life for infants suffering perinatal brain injury.

Keywords Neuroprotectant · Melatonin · Behavioral testing · Preclinical

Introduction

Within this review, we will outline what we consider the key stages of preclinical testing and clinical trial development for a neuroprotectant or regenerative neurotherapy for perinatal brain injury, illustrating in particular the key preclinical analyses that can facilitate expedient movement through the testing pipeline. We will for the most part discuss the testing and development of melatonin as an example neuroprotectant. We will trace its testing in reductionist small animal models to more complex, larger and gyrencephalatic preclinical testing models and the current clinical trials. We will also highlight examples of new small animal physiological and behavioral testing that gives small animal preclinical models greater clinical relevance.

Perinatal Brain Injury

Definition and Incidence

Perinatal brain injury in term and preterm infants is associated with lifelong sensorimotor and cognitive disabilities.

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The causes of perinatal brain injury are multifactorial and include infection/inflammation, prematurity, stroke, asphyxia, genetic defects, and intrauterine growth restriction [1–4]. The incidence of perinatal brain injury is not decreasing despite substantial advances in perinatal care; incidences are as high as 3 of 1,000 live births in first world countries but rates are devastatingly higher in developing countries (6–14 of 1,000 live births) [5]. Gestational age is a key factor in the distribution of injury, with white matter injury predominating in the preterm infant and grey matter tending to be more common in the term infant. However, some amount of co-incident grey and white matter remains a component of injury in all age groups [6–8].

Treatments

Clinically, the acute and secondary pathological processes of brain injury cannot be treated in the preterm infant. In term neonates, therapeutic hypothermia applied to infants diagnosed with hypoxic–ischemic encephalopathy (HIE) based on Apgar score, pH, and lactate levels is able to reduce secondary energy failure and reduce mortality at 18 months [9, 10]. However, a meta-analysis suggests that improvements in morbidity long term might be limited, requiring further anal-ysis of the clinical data [11]. Many alternative and additional treatments are being tested, but they have so far shown only limited safety and efficacy. It is outside the scope of this review to discuss these fully, and we refer the reader to thorough reviews on this topic published elsewhere [12–14].

A variety of interventions (see [13, 15]) have proven efficient to treat brain injuries in adult animals, although these have not translated well into the clinical setting. However, even in the experimental setting, direct extrapolation of these therapies to the neonatal brain (including the choice of therapy and specific treatment regime) is confounded by substantial differences in damage and repair mechanisms between the neonatal and adult brain [16, 17]. We suggest that a promising strategy for identifying novel neurotherapeutics consists of testing drugs already approved for use in pathologies of other organs, such as those on the Food and Drug Administration (FDA)-approved drugs list or even those with limited efficacy as adult neurotherapies. However, to expedite this process, we need to understand the positive and negative facets of our research efforts to date to translate therapies from reductionist animal models through to clinical trials. Importantly, we believe greater emphasis must be put on high throughput animal models for screening drugs and a strict pipeline of increasingly complexity and clinical relevance to the human condition to expedite this process.

Animal Models of Perinatal Brain Injury

There are advantages and disadvantages to every type of animal model, in both biochemical similarity to the human and related to cost and practicality of husbandry. In relation to large or small animal models, benefits of small animals models includes the ability to test a wide range of doses, treatment regimes, administration routes, and outcome times as well as testing complex neonatal and adult behaviors. However, rodents (and other smaller animals) are typically lissencephalic and have various biochemical and physiological differences to humans. The greatest contrast between these two generalized groups (large vs. small) is the length of gestation and cost, per animal and in housing—which is far greater in larger animals. Benefits of using large animals (i.e., piglet, sheep, and nonhuman primates) include monitoring a great number of basic physiological variables (blood gases, EEG, respiration, heart rate, and temperature), physiology, and gyration. However, substantial improvements in imaging and the development of testing apparatus for the basic physiology of rodents are increasing the utility of small animal models as a viable preclinical tool (*discussed in full below*). Also, as mice are the preferred mammalian species for genetic studies, methods for behavioral mouse phenotyping are important instruments to investigate the molecular targets of treatments. Models in rodents are typically considered higher throughput that those utilizing sheep and piglets—as with models such as the mouse ibotenate acid model of neonatal excitotoxic lesion a highly reproducible injury can be induced in many tens of animals in a morning. Experiments involving brain lesions in a similar numbers of sheep or piglet studies would take many months.

Brain injury in perinates can be initiated by, and can be the cause of a cascade of various injurious processes, such as inflammation, excitotoxicity, and hypoxic–ischemic events. Thus, only by examining the efficacy of therapies in multiple models of different injury types and identifying key similarities can we identify clinical efficacy. There have been numerous well-considered and thorough reviews of the various types of animal models of perinatal brain injury, and we refer the reader to these for details on the relevant similitudes and differences relative to the human condition [18–21]. We will provide only a brief outline in tabular form of the many animal models of perinatal brain damage (Table 1), adapted from [19, 21] to orientate the reader on the current models, especially those later mentioned in this review.

Melatonin, a Treatment of Choice?

Melatonin has been tested as a neuroprotectant in numerous types of brain injury models including those involving inflammation, hypoxia, ischemia, trauma, excitotoxicity, and malnutrition in many species including rodent, rabbit, piglet,

Table 1 A selection of commonly used models of perinatal brain injury

Model	Species commonly used	Effects of melatonin studied[a]	Representative developmental stage	Pathological features and selected general observations	Selected references
Acute hypoxia/ischemia	Mouse or rat	Yes—protective	Preterm or term dependent on age of pup (P1–P7)	Microglial activation Ventriculomegaly Axonal damage More severe when coupled with inflammation Preterm(P3–P5): Oligodendrocyte cell death—Alterations of oligodendrocyte development—Necrotic cysts of white matter and cortex Term(P7+); Predominance of forebrain neural cell death	[22–25]
	Piglet	Yes—protective	Term	Cell death in multiple forebrain structures (cortex, thalamus, and caudate) and white matter tracts (internal capsule and periventricular white matter. Altered magnetic resonance imaging (MRS); increased lactate and decreased ATP	[26–29]
Chronic hypoxia	Mouse or rat	Yes—protective	Preterm or term dependent on age of pup (P1–P7)	Oligodendrocyte cell death is minimal Alterations of oligodendrocyte development Moderate neural cell death Ventriculomegaly	[30–33]
In utero asphyxia	Rabbit	Not trialed	Term	Lesions to multiple forebrain structures (cortex, thalamus, and basal ganglia) and white matter tracts Intraventricular hemorrhage	[34]
	Sheep	Yes—protective	Preterm but variable depending on fetus (50-75 % GA)	Relatively selectively graded cerebral white matter lesions that resemble the spectrum seen in human Cortical and subcortical gray matter injury is a major feature if ischemia is severe Very similar to LPS insult in fetal sheep	[35–38]
	Spiny mouse (*Accomys cahrinus*)	Yes—protective	Term	Microglial activation Limited oligodendrocyte cell death Moderate neuronal loss Astrocyte cell death	[39, 40]
	Macaca nemestrina (pigtailed macaques)	Not trialed	Term	Increased lactate and Apgar <3 at 10 min resuscitation. Slowed weight gain, early deficits in motor development and memory.	[41, 42]

Table 1 (continued)

Model	Species commonly used	Effects of melatonin studied[a]	Representative developmental stage	Pathological features and selected general observations	Selected references
				Slightly smaller cerebellum at 2 years. Negligible effects of asphyxia on MRS	
Premature delivery	Baboon	Not trialed	Preterm	Cystic necroses Diffuse white matter gliosis Ventriculomegaly Intraventricular hemorrhage	[43, 44]
Acute inflammation	Mice, rats, sheep, and rabbit	Yes—protective	Preterm or Term dependent on age of animal	Microglial activation Diffuse white matter damage including decreased myelination Oligodendrocyte cell death in severe inflammation models Deep cortical layer neuronal cell death in severe inflammation models More severe when coupled with HI	[45–49]
Chronic inflammation	Mice and sheep	Yes—protective	Preterm or term dependent on age of animal	Severe and term equivalent inflammation: decreased proliferation—deceased myelination—widespread injury in the periventricular and subcortical white matter and grey matter—microgliosis—acute loss of astrocytes and regional infiltrates of inflammatory cells Moderate and preterm equivalent inflammation: delay of oligodendrocyte maturation—axonapathy—decreased myelination—very limited cell death—transient microgliois—reduced learning	[50–52]
Excitotoxic	Mouse and rat	Yes—protective	Preterm or term dependent on age of pup (P1–P11)	Predominance of white matter injury at P5–7 Greater cortical damage P10–11 Microglial activation Astrocyte cell death Loss of oligodendrocytes limited and only in infarct core Reduced scores in neonatal and adult cognitive tests	[53–57]

MRS magnetic resonance spectroscopy, *P* postnatal day

[a] In at least one variant of the model

chick, and sheep, modeling the multiple forms of white and grey matter deficits of the term and preterm human infant. Across these models, melatonin has been shown to be neuroprotective and safe (indicated in Table 1).

Melatonin (5-methoxy-*N*-acetyltryptamine) is an indoleamine produced by the pineal gland in response to light–dark cycles and also by the skin, retina, gastrointestinal tract, and lymphocytes in animals [58–60], and interestingly, it is also produced by both plants and unicellular organisms, see [61]. Melatonin is lipophilic and as such diffuses through biological membranes and easily crosses the blood brain barrier. Melatonin is a potent antioxidant [62], this free radical scavenging role suggested to contribute to its anti-aging effects [63]. Melatonin also regulates immune function (see [64]), and decreases in its production during summer and associated with aging is thought to underpin our seasonal susceptibility to disease and immunosenescence (see [64]). Melatonin reduces apoptosis by stabilizing the mitochondria [65, 66], and it also reduces proliferation, a property underpinning its antioncogenic properties [67].

There are two well-characterized membrane-bound melatonin receptors ($Mel1_a$ and Mel_{1b}), both G-protein coupled receptors, distributed throughout the brain, immune system, and almost all other organs and tissues (see [68]). Melatonin also acts via orphan receptors, nuclear receptors, or via interactions with molecules, such as calmodulin [69]. The nonreceptor-mediated actions of melatonin are predominantly antioxidant.

Melatonin has been used for many decades to treat sleep disorders and seizures in adults and more recently in children and young infants, with great success and with very limited safety concerns [70, 71]. Melatonin has been used successfully in very small clinical trials in infants to reduce oxidative stress and septic shock and the incidence of bronchopulmonary dysplasia [72–74]. These human data together with the wealth of experimental evidence has developed a strong case for full-scale clinical trials, including long-term follow-up, to fully quantify the protective capacity of melatonin in the infant brain.

However, before melatonin even reached this point, there were pilot studies, failed (and perhaps unpublished) experiments, and often the necessity to repeat smaller studies to create cohesion and reinforce data. In our endeavor to create a therapy to protect/repair the brain, we must search for ways to reduce the burden of these false starts. As even if melatonin can join hypothermia as a tool for improving brain health long term, even the most optimistic of us can see we are still far from having all the tools we need.

Idealized Stages in a Pipeline Trialing a Neurotherapeutic

It is implausible to present a complete instruction manual for the testing of a novel neurotherapy. However, using melatonin as an example where possible, we will illustrate in this review many of the key experimental milestones and outcomes that would in our opinion form part of an ideal world scenario of a drug trial pipeline. A critical concern in animal models is increasing clinical relevance, while facilitating high throughput. As such, we will focus in the last section on the improvements in small animal physiological monitoring and testing that we hope will substantially improve the efficacy of trials, bringing us sooner to the end goal of improved clinical outcomes. In this section, we will discuss briefly clinical trials, the regulatory aspects governing pediatric drug trials, and models of injury. These injury models range from reductionist through to complex models with comorbidities and those involving non-human primates. Altogether, we hope to provide a context for the most expedient manner to test novel neurotherapeutics.

Clinical Trials: Keeping the End Goal in Sight

It is important to recognize that the justification for the clinical trial of any therapy is a solid body of evidence of improved outcomes (histolopathological, functional, and imaging based) across complex pre-clinical models, clinically feasible administration route and formulation, and a favorable safety profile. Melatonin is currently the focus of several clinical trials, (1) administration daily for 7 days after premature birth to identify if it may reduce the risk of prematurity-associated brain injury (MINT; ISRCTN15119574), (2) investigation of the physiological secretion of melatonin in the premature and full-term baby to identify optimal treatment doses (MELIP; NCT01340417 and MIND, NCT01340417), and (3) the effects of maternal supplementation on outcome in term infants (PREMELIP; ID pending). Melatonin has also been granted orphan status for the treatment of perinatal asphyxia by the European Medicines Agency in February 2012 (EMA/OD/133/11), an important endorsement of the possible clinical importance of melatonin as a therapeutic.

Given this wealth of activity, we await with great interest enough evidence to make a considered decision on the efficacy and long-term safety of melatonin for treating perinatal brain injury.

Regulatory Aspects of Pediatric Drug Testing

Within the field of noncommercial perinatal research, such as universities and hospitals, new candidate neuroprotectant compounds have been typically selected from within previously FDA-approved drugs. Use of FDA-approved drugs removes much of the necessity for basic safety testing, which is both enormously costly and labor intensive and which has been described in detail elsewhere [75, 76]. However, this testing has in the past not specifically included infants or neonates and basic physiology varies

dramatically and may mean that little is known about the impact of a drug across organ systems in the developing animal. As such, in the last years, a series of developed countries have adopted new legislative and regulatory frameworks for pediatric drug development [76]. Considering these rules is both informative and helpful in translating a novel therapy from the bench through to the cribside.

Pediatric assessments are required as part of every new drug application and biologics licensing application in the USA, and every marketing authorization application in Europe (see [76]), unless a specific waiver has been granted. Pediatric investigation plans are generally expected by the European Medicines Agencies when adult pharmacokinetics data are available (normally end of phase 1). In the EU, regulation No. 19012006 on medicinal products for pediatric use covers both the mandatory evaluation of new products as well as mechanisms for a 6-month extension of the supplementary protection certificate.

Both FDA [77], and the European Medical Agency [78], produced guidance documents that address the importance of conducting preclinical safety studies in developing mammals with special consideration for organ systems that undergo significant postnatal development. Furthermore, a new law on pediatric drugs adopted by the European Parliament in 2007 and the European Agency for the Evaluation of Medicinal Products (EMEA) has emphasized the need for preclinical studies in developing mammals (EMEA Guidelines). Thus, as we have improving technology (see "Monitoring Clinically Relevant Physiological and Behavioral Impairments"), physiological, psychomotor, and cognitive testing in newborn mice represents a useful tool in these preclinical studies of effectiveness and toxicity of pediatric drugs.

Juvenile animal studies have also been promoted as potential better predictors of toxicity in pediatric patients than the standard testing in adult animals, based on the previous unanticipated toxicities being observed in pediatric patients in the clinic [75]. This supports the design of preclinical study to evaluate all potential outcomes, rather than only the effects anticipated from adult studies. Thus, developing cognitive tests for newborn and juvenile mice is a priority challenge for neurogenetics and pharmacological research.

High-Throughput In Vivo Screening: Reductionist Modeling of Perinatal Brain Injury

Key safety and efficacy data to justify clinical trials is based on large preclinical animal studies. Importantly, the rationale and funding opportunities to complete these large preclinical studies is often based on thorough studies in reductionist high-throughput small animal models. Thus, well-considered small animal/reductionist animal model trials are a critical 1st stage in the pipeline of therapeutic drug validation (Fig. 1). Reductionism allows specific aspects of a complex injury to be investigated, such as the excitotoxic component of hypoxic–ischemic or inflammatory injury and/or the exclusion of the effects of multiorgan dysfunction (discussed below). Coupled to animals with a short gestation and lower husbandry costs, this approach can facilitate screening of drugs and the justification for research in models of increasing complexity, cost, and ethical considerations.

Excitotoxicity is a common component of brain injury due to inflammation or hypoxia/ischemia (HI), commonly suggested effectors of perinatal brain damage. Excitotoxic lesions in the periventricular white matter and overlying cortex of neonatal mice can be produced quickly and reproducibly, providing a highly useful model for screening. Using this reductionist model of perinatal brain injury, Husson et al. [55] were among the first in the perinatal field to illustrate the neuroprotective ability of melatonin and its potential clinical applicability; included in their paper are *dose response*, *basic physiology*, *administration route*, a time course of *delayed treatment*, and a *short- and longer-term assessment of outcome*. Thus, this study contained many of the key facets important in screening a novel neuroprotectant and all together demonstrated that melatonin was efficient in reducing excitotoxic white matter lesion size in the microgram-per-kilogram range but caused a small reduction in body temperature. However, melatonin had efficacy even when administered systemically, could be given with a delay of up to 4 h after injury, and the lesion was still reduced 2 weeks after injury. This paper also began investigating the *mechanism of action*, showing protection was dependent on the receptor-mediated effects of melatonin. This study, and a later more mechanistic study into the protective role of melatonin after chronic hypoxia [33], point to an important feature of a well-performed study, the *inclusion of multiple brain regions in an analysis*, as, melatonin in these two models was shown to be protective only in white, and not grey matter. However, grey matter protection has been shown with melatonin treatment in other animal models [36, 79], and following repeated administration in this model (*see below*).

Husson et al. [55] did not consider the *delayed therapeutic window* for melatonin administered directly into the cerebrum, although i.p. treatment could be delayed by 4 h and still be highly effective. In a follow-up study, a single dose of melatonin i.p. had to be administered within 8 h to have any neuroprotective effects. However, delaying treatment to 24 h was protective when treatment was repeated (in this case a total of five times), every 24 h. Furthermore, this regime also leads to a reduction in grey matter injury (not only white matter) that is not seen with early/single-dose treatment only (Fig. 2), thus illustrating the importance of trialing multiple *treatment regimes*.

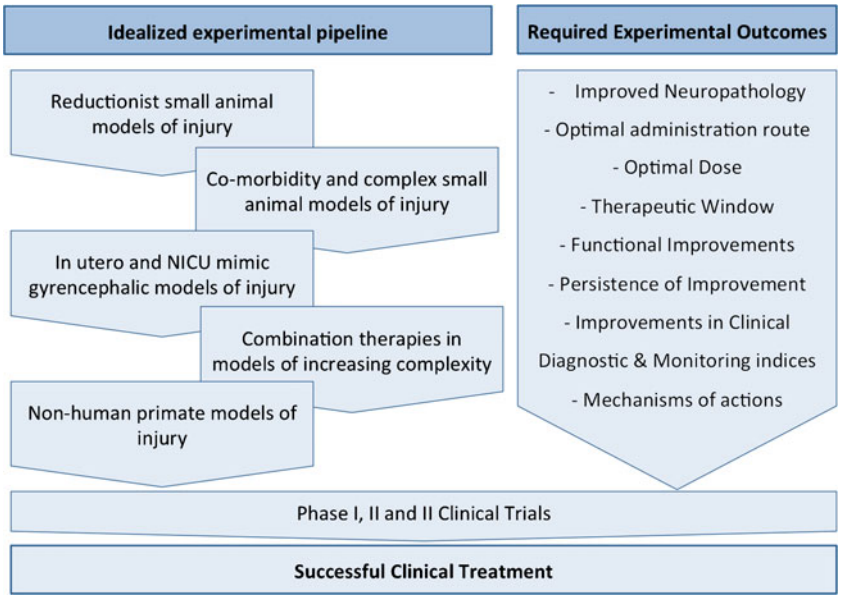


Fig. 1 The basic stages in an idealized experimental pipeline of drug validation, and in parallel the experimental outcomes required to be validated at each step within the pipeline

Further to this study, completing the characterization of the effects of melatonin in this excitotoxic model, a battery of behavioral tests has been performed, discussed in full below, in "Monitoring Clinically Relevant Physiological and Behavioral Impairments."

Another study attempted to characterize further the *administration route* and *window of efficacy* of melatonin, utilizing an in utero model of global term equivalent asphyxia in the precocial spiny mouse (*Acomys cahirinus*).

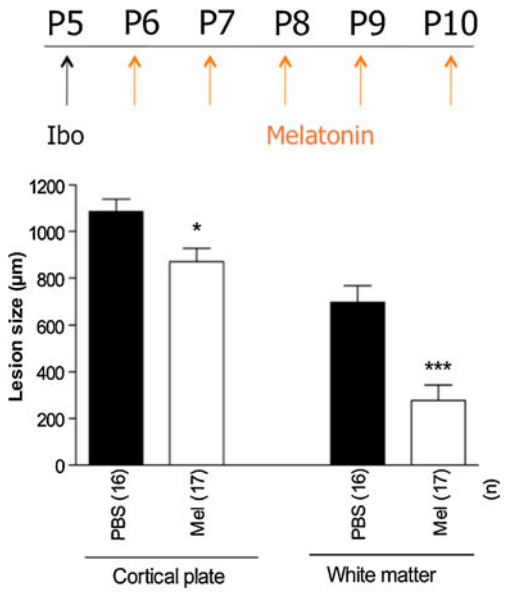


Fig. 2 Delayed therapy with melatonin reduces excitotoxic lesion size in neonatal mice. Schematic representation of the treatment regime with ibotenate lesion induced at postnatal day (*P*) 5 and daily injections of 5 mg/kg melatonin given once immediately after injury and then once daily for 5 days. Melatonin reduced lesion size in the cortex and white matter. Values are mean±SEM. Data analyzed with student's *t* test. *$p<0.05$; **$p<0.01$; ***$p<0.001$

Melatonin was administered in the drinking water for the last 20 % of gestation [39]. Of note, melatonin levels were measured and found to be increased in the fetal plasma and brain before the insult, and cell death and gliosis induced by the asphyxia were reduced. However, melatonin increased overall fetal brain weight and decreased below control levels expression of activated caspase-3. This potential effect of melatonin on developmental apoptosis highlights the importance of monitoring the *developmental effects of therapies* elucidating not only the window of efficacy but also the window of safe administration of any treatment.

The Rice-Vannucci model of HI [22], is also an invaluable reductionist model of the human infant exposed to HI, (e.g., due to placenta previa or tight nuchal cord). Variants of this protocol of carotid artery ligation and exposure to hypoxia in rats and mice of varying ages are used to recapitulate injury patterns seen in the term and preterm infant. Interestingly, this model removes the variable of maternal-fetal signaling inherent in in utero models. Furthermore, rodents have functional control of breathing despite the immature (altricial) nature of the brain at birth. As such, utilizing this postnatal model removes the necessity to ventilate (technically very difficult in newborn rodents), which is often suboptimal and leads to inflammatory pulmonary damage [80]. Using this well recognized model Carlonini and colleagues demonstrated that melatonin reduces infarct volume shortly after injury [81]. Importantly, they also demonstrated that *adult behavior* was improved, including long-term retention (15 day) of complex water maze learning (*discussed further below*).

Increasingly, technological advances mean that these types of small animal studies can be supported by clinically relevant imaging techniques, including magnetic response imaging, such as, fractional anisotropy assessed with tract-based spatial statistics, magnetic resonance spectroscopy for biochemical

changes, and traditional T1 and T2 imaging of tissue density available at up to 12 T. Ultrasound technology is also greatly improved with blood flow measurements and even task-evoked brain activation now possible with high spatiotemporal resolution in small animals [82]. These techniques allow monitoring of the evolution of injury and treatment efficacy, and their application in conjunction with basic histopathology is critical for our understanding of how we identify and treat infants, in whom imaging is our only window into the pathological events in the brain. However, as mentioned further below advances in co-registration are needed for us to identify how changes in our imaging signals directly correlate with cellular and subcellular changes in the brain tissue.

Modeling Brain Injury in Global Insult, Precocial and Gyrencephalic Animal Models

Enormous improvements in rodent physiological and clinically applicable testing practices mean we can acquire a great deal of useful data in easy-to-handle and high-throughput small animal models. Strong positive data are then a stepping stone to trial drugs in models with a greater number of characteristics with similarity to the human condition, such as: a global insult, the stress of fetal extra-uterine transition, that have complex cortical folding, allow an in utero injury or can be maintained in a NICU environment.

Two papers have described this type of animal model (i.e., global intra-uterine insult and gyrencephalic), the fetal sheep model of preterm intrauterine HI to illustrate a beneficial effect of melatonin administered directly to the fetus, at mid- [36] and late gestation [35]. These studies were performed almost a decade ago and using the ability to chronically instrument the lambs facilitated detailed physiological and biochemical analysis of the response to insult and treatment (including cerebral microdialysis), important data not accessible from small animals at that time.

In our 2012 ideal world scenario, to derive maximal utility from scaling up the complexity of the experiment model to the fetal sheep, clinical imaging techniques would now also be applied [38], EEG monitored [83], and importantly levels of melatonin measured to facilitate translation of effective doses across species, such as has been done recently in a piglet neuroprotective trial involving melatonin [84]. Interestingly, behavioral analysis comparable to an Apgar score can also be performed on lambs [85, 86] and more complex testing has been developed for piglets [87], possibly adding greatly to the clinical relevance of this type of large animal studies.

The Final Pre-clinical Step: Primate Models of Perinatal Brain Injury

In this idealized pipeline, following positive outcomes in models of increasing complexity and similarity to humans, in our opinion well-considered studies in primates represent an important final preclinical transition. We feel this step is critical for two reasons, firstly, due to biochemical parameters that differ between experimental animals and humans, including enzymes such as lactate dehydrogenase and superoxide dismutase [88, 89]. Secondly, that NICU care often involves pharmacological treatments to maintain homeostasis and noxious stimuli, which may interfere with the efficacy of therapies, an effect that to date can be best mimicked utilizing the nonhuman primate. However, we acknowledge that differences exist between non-human primates and humans. Of note, hypothermia has become best practice in treating term infants with a diagnosis of HIE, without testing in nonhuman primates. However, given that this is a procedure not a drug, perhaps species-specific biochemical variations have less impact in translating such a therapy.

Despite the benefits of utilizing primates, these types of experiments are ethically challenging, expensive, time consuming, and expertise heavy. Comprehensive and conclusive experimental data from nonprimate models of administration route, dose, therapeutic window, and persistence of improvements must be used to formulate experiments with nonhuman primates. Also, the experiments must be designed to maximize the utility of any data in planning clinical trials, including detailed analysis of basic physiology and outcome, as imaging correlated to histopathology and behavior.

In our idealized pipeline, we can consider two models currently in use for perinatal brain injury in nonhuman primates, the baboon model of preterm birth and the macaca model of perinatal asphyxia [41, 90]. The baboon model recapitulates the white matter injury characteristic of preterm infants, via delivery of the baboons at 0.75 of gestation followed by two full weeks of full-time NICU care. This model is primarily designed to test the efficacy of modes of ventilation and therapies to prevent lung damage. Despite this, studies from this group capture many of the facets that must be monitored in an ideal study of a neuroprotective in nonhuman primates and provides interesting data to the field on these compounds. *Basic physiological data* and *detailed brain volume and weight* measurements were collected in studies of the effects of estrogen and inhaled iNOS on brain development subsequent to the use of these compounds to reduce lung injury [91, 92]. Analysis was made of *multiple indices of brain structure/function* (gliosis, white matter injury, vasculargenesis, and proliferation) and cell death across *multiple brain regions*, including the cerebrum and the cerebellum. These data demonstrate no effect of either treatment on the brain. This may be unsurprising as the neuroprotective efficacy of estrogen in smaller animal models of injury is not completely certain [93, 94], and although iNOS is effective in reducing injury in rodents [95, 96], there is no study that we are aware of in larger and gyrencephalic animals.

The use of the baboon for modeling for lung injury may explain the absence of imaging in these aforementioned studies, which we consider a critical component of primate studies. However, a characterization of the prematurity-induced brain injury in this model using MRI and immunohistological staining was conducted, which attempted to *correlate clinical and histopathological observations*. A limitation of this study, and many studies combining imaging and histopathology, is a lack of co-registration of the imaging data with the tissue sections. Due to the many deformations caused within the brain following imaging, during the procedure of processing and preparation for staining, without co-registration areas of interest identified can only represent approximations [97–99]. Thorough co-registration is difficult and time consuming, but it is critical that we begin to fully understand the relationship between signal intensity changes and histopathology. To identify mechanisms of injury and to monitor improvements in the brain, we must gather information on the physiological basis of our imaging proxies.

Melatonin has been used safely in pediatric medicine to treat seizure disorders for many years [70]. Based on this safety profile, small clinical trials using melatonin have been performed in infants. These have also demonstrated the safety of melatonin (even at high doses, i.e., 100 mg over 72 h) and importantly efficacy in reducing poor outcome in infants with asphyxia and sepsis [72, 73]. It may be considered that these studies together with the wealth of animal data on safety and efficacy have lead to initiation of large-scale clinical testing without primate experiments. However, in this idealized pipeline, additional safety and efficacy data from primate experiments would be an important pre-clinical step.

Modeling the Complexity of the Human Condition in Simple Animal Models: Comorbidity, Gestational Age, Pain, and Sex

The varied and often complex comorbidities in adult patients affected by brain injury (such as atherosclerosis, obesity, and diabetes) are considered to be a key reason that highly effective treatments for insults, such as stroke have failed to translate from animal models to the human population [100, 101]. In the neonate, it may be argued that there is less comorbidity, however, suboptimal genetic traits (polymorphisims), inflammation (due to maternal obesity or infection), placental insufficiency, sex, and stage of development are key variables. In addition, infants with perinatal brain injury, (especially premature infants) commonly experience multiple episodes of pain and stress, inescapable realities of life-saving procedures within the intensive care unit [102, 103]. Furthermore, they are exposed to drugs, such as morphine, fentanyl, midozalam, dexamethasone, and general anesthesia. Anesthetics, stress, and pain can cause damage or alter programs of development in the brain [104]. Conversely, anesthetics can also be neuroprotective in specific context [105–107], perhaps important in reducing the deleterious effect of co-incident noxious events around injury and treatment [108]. As such, when modeling perinatal brain injury, consideration should be given to the possibly synergistic or sensitizing effects of these types of factors on modes of action or implementation of therapies.

As such, in our idealized pipeline, we must ensure the (often persistent) noxious environment of the NICU does not abrogate the therapeutic effects of any treatment. The piglet models of perinatal asphyxia undertaken by Robertson et al. and by Thoresen et al., are valuable (nonprimate) term equivalent models to asses this, allowing for up to 48 h of NICU care, including supported ventilation, administration of morphine, fentanyl and isoflurane, maintenance of cardiac output, blood volume and electrolytes, cardiac resuscitation (if required), and continuous EEG monitoring [26, 106, 109]. Robertson et al. have recently used this model to test the efficacy of melatonin to augment hypothermia [84] and how this study has added to the data for translating this therapy into clinical trials is described below, under combination therapies.

Considering the need for neurotherapeutics to overcome injury due to insult and the noxious events inherent in the NICU, an earlier study addressed the specific question of whether melatonin could reduce cell death in a term equivalent rat model (P7) exposed to the clinically relevant combination of midazolam, isoflurane, and nitrous oxide [65]. A *dose-dependent* reduction in cell death was seen across *multiple brain regions*, and interestingly the *mechanism of action* was described to include countering the anesthesia-induced loss of BcL2, decreasing mitochondria-dependent apoptosis. Furthermore, in both adult and neonatal models, melatonin reduces respiratory inflammation, associated with supported ventilation [110, 111].

That sex effects outcome after injury to the adult brain has been appreciated for many years due to epidemiologic and experimental studies [112, 113]. As these effects were primarily ascribed to be sex hormone dependent, it took far longer for the importance of sex in the perinatal period to be recognized as being an important factor in outcome, males at an increased risk for mortality and morbidity [114]. We now appreciate that there are considerable sex differences in gene expression from early embryogenesis [115, 116], and persisting differences in injury processes [117, 118] and cell death mechanisms [119, 120]. As such, it is unsurprising that trials of neurotherapeutic candidates also report sex differences in the efficacy of treatments for the neonatal brain [95, 121]. It is likely that the proportion of sex-dependent therapies is far greater than reported as many trials fail by considering male or pooled data only. As such, although historically an undervalued

variable in perinatal research, it is critical to consider sex when trialing neurotherapies.

Inflammation is almost ubiquitous in cases of perinatal brain injury, independently considered a causative factor and also to be induced by other causal factors including infection, trauma, and HI. Inflammation also sensitizes the brain to subsequent injury, whether in the perinatal period or even into adulthood [46, 122–125]. It is however, often unknown when inflammation is initiated in the infant brain and whether it represents the cause of an injury, or is the result of an insult. However, as it is (as yet) impossible to determine when inflammation begins in the human population unequivocally, it is important that we show efficacy in treatments with various permutations of inflammation and additional insults (further inflammation, prematurity NICU stress and hypoxia) to insure we can offer effective neuroprotection. With this in mind, we can note that melatonin reduces excitotoxic lesion size in the immature brain in a model without inflammation or when the pro-inflammatory cytokine IL1b is used to induce inflammation prior to the injury [49].

In addition, exercise, environmental enrichment, and tactile stimulation are known to improve outcomes after brain injury in rodents [126–128] and improve outcomes in infants and adults with brain injury [129]. As such, our treatments must be able to improve outcome more than these therapies in the infant. More speculatively, perhaps we must consider that a portion of the current failure of therapies to translate into the clinic might be due to their efficacy only in an environmentally deprived context [127].

Combination Therapies

Multiple injurious pathways are activated by the most common brain insults and cause perinatal brain injury, such as reactive oxygen species, inflammation, and excitotoxicity. Thus, in the ongoing hunt for efficacy, it is increasingly common for therapies targeting different pathways to be combined. This is particularly true for hypothermia (see [130]) which as the only effective therapy against neonatal brain damage, is routinely implemented in the majority of first world hospitals in term infants (36+weeks gestational age) presenting with low Apgar score, high lactate, and low blood pH [131, 132].

Within the idealized testing pipeline, combination therapies should in many regards be considered a completely new therapy. In as much, repeating in reductionist through to complex models assessments of dose responsiveness, the therapeutic window, histopathology, physiological parameters, and clinical indices of outcome are required. In the case of hypothermia, it itself may alter the pharmacokinetics and efficacy of drugs due to its overarching effects on metabolism [133, 134]. As such, it is critical to consider a noncooled control and noncooled treatment group to ensure that we are fully aware of the potential benefits and pitfalls of all therapies.

In a complex animal model of term perinatal asphyxia, the piglet (see above, "Modeling the Complexity of the Human Condition in Simple Animal Models: Comorbidity, Gestational Age, Pain, and Sex"), melatonin has been shown to augment the effects of hypothermia [28]. Interestingly in this study, *clinical diagnostic imaging* with magnetic resonance imaging demonstrated increased energy reserves and decreased lactate in hypothermia plus melatonin treated piglets. *Histopathology* revealed this effect generally correlated with decreased cell death. Also, relatively uncommon in large animal models, there was a *dose response* to validate the safety profile of the treatment, and this study attempted to identify a *mechanism of action* for neuroprotection, assessing oxidative stress and microglial phenotype. With large animal models, multiple time points are difficult, but at 48-h postinjury, it is suggested that an immunomodulatory effect of melatonin, but not its antioxidant properties improve imaging and histopathology, while cardiovascular function was undisturbed by treatment.

Mechanistic Studies

Detailed mechanistic studies can be the precipitating factor for trialing a therapy, or the second step in understanding and refining a therapy. Mechanistic studies often utilize in vitro techniques and these observations have immeasurable utility. However, within this idealized pipeline, we must ensure studies include a final confirmation in the whole immature brain of any observation to validate any biological relevance for the infant. Mechanistic studies are not critical for the development of a neurotherapy, but of course for identifying our ability to refine and best implement any treatment understanding its mechanism of action are of paramount importance. Of note, there is a substantial amount of research on the effects of melatonin across adult injury models and work into the mechanism of action upon which research into its effects on perinatal brain injury was founded. The mechanisms of neuroprotection for melatonin have in brief, been categorized as being able to stimulate postlesional plasticity [49], as an anti-oxidant and anti-inflammatory [135], and as an anti-apoptotic [66]. These, and the many other mechanism of actions for melatonin have been thoroughly reviewed elsewhere [61, 62, 64, 136].

Monitoring Clinically Relevant Physiological and Behavioral Impairments

Learning deficits are a hallmark of the later neurodevelopmental disorders in infants suffering perinatal brain injury [11, 137]. Specifically, children with white matter injuries display deficits in learning, memory, attention, and

intellectual performance [138, 139], and verbal associative learning is altered in adults born very preterm [140]. Furthermore, gait disorder is one of the main characteristics of children with white matter diseases, such as cerebral palsy [141]. Importantly, disability scores at 30 months of age were found to be very good markers of disability at 6 years of age [137], thus stressing the importance of early identification to facilitate interventions.

The development of methods for assessing behavior in adult mice has received considerable attention and has lead to methods of assessing multiple forms of complex learning and memory defects [142–145]. In sharp contrast there are very few methods for behavioral assessment during early development, especially in newborn mice. Cardiorespiratory and thermoregulatory functions cannot be assessed in newborn mice using commercially available devices. Unfortunately, previously reported homemade systems that are currently used for research on these functions are poorly suited to high-throughput assays. To date, the only well-established, robust, and relatively sensitive method for psychomotor development is the Fox Battery [146], which includes a series of tests that tap various aspects of

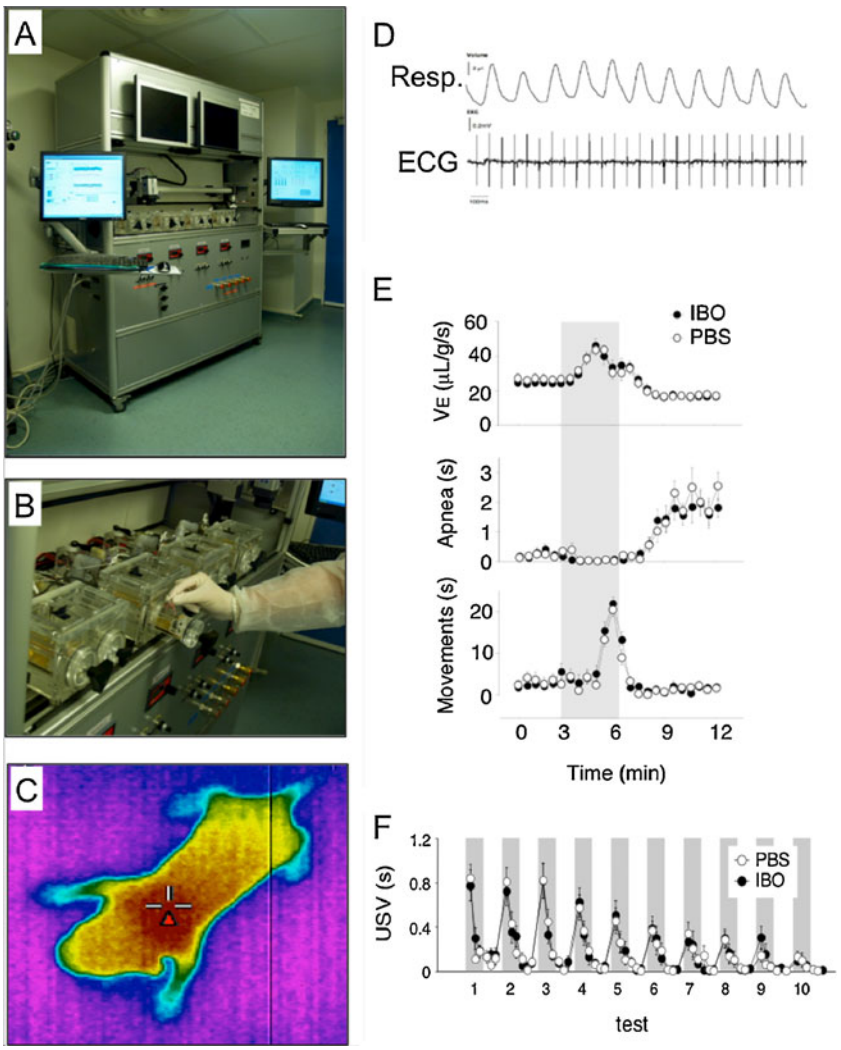


Fig. 3 High-throughput screening of physiological functions in newborn rodents. **a** General view of the physiopups system, for simultaneous and noninvasive measurements of breathing pattern, heart rate, body temperature, ultrasonic vocalization, and gross body movements in newborn mice (or rats). Four pups can be tested simultaneously. **b** Each pup is installed in the chamber in which it may move freely. Temperature and gas composition in the chamber are controlled, and experimental protocols and data collection are performed automatically. **c** Body temperature is assessed by infrared thermography of the skin over the interscapular region containing heat-producing brown adipose tissues. **d** Examples of respiratory and electrocardiographic traces in a 2-day-old pup. **e** Comparison of pups with ibotenate-induced brain lesions (*filled circles*) versus PBS-treated controls (*empty circles*). Values are means±SEM [154]. Ventilatory and motoric responses to a hypoxic challenge (5 % O_2 in N_2, *shaded area*) in brain lesioned in 6-day-old mouse pups and their PBS controls. The induction of excitotoxic lesion mice had no significant effect on ventilation (V_E), apnea duration and defensive movements, and ventilatory and defensive responses to hypoxia. **f** Reactivity to tactile stimulation in brain lesioned in 6-day-old mouse pups and their PBS controls. Tactile stimulation provoked similar ultrasonic vocalizations in ibotenate-induced brain lesions (*filled circles*) and PBS-treated controls [154]

psychomotor development: righting reflex, postural flexion and extension, cliff aversion, negative geotaxis, etc. However, these tests do not provide direct information on the ontogeny of gait, which is a main focus of interest with respect to early neurological disorders in human infants.

The analysis of emotional responses in developing rodents, especially mice, is based on the analysis ultrasonic vocalizations (USV). In newborn mice, USV have been studied both as an early communicative behavior with the mother and as a marker of an aversive affective state usually regarded as anxiety [147, 148]. Of note, the notion that mouse USVs in infant rodent are similar to cries is debated (e.g., [149]). Mouse pups emit USVs when removed from the litter and in response to cold. USV prompts the mother to retrieve the pup to the nest. The communication function presumably served by USV make their study particularly relevant to the modeling of autistic disorders in mouse pups (see [150]).

Learning procedures developed in adult rodents, are not suitable for testing newborn rodents, because of the newborn's motor and sensory immaturity. Furthermore, learning in newborn mammals differs from learning in adults: newborns have far greater capacity for learning but less capacity for memorizing [151]. The development of learning and memory in mouse pups is mainly investigated through abilities to form associations. Because of the limited behavioral repertoire of newborn rodents associative abilities have been assessed using novel odors as conditioned stimuli and food or stroking as the unconditioned stimulus (reviewed in [152]). While these studies have been mostly conducted in rats, these methods have been recently extended to mice, and generalized using thermal instead of stroking stimuli, as detailed below. A platform for assessing vital functions, psychomotor skills and cognition has been developed at Inserm U676 Robert Debré Hospital in Paris, which to date is unique its ability to provide such a comprehensive set of behavioral and physiological outputs (*discussed further below*).

Idealized Battery of Physiological and Behavioral Preclinical Testing in Newborn Rodents

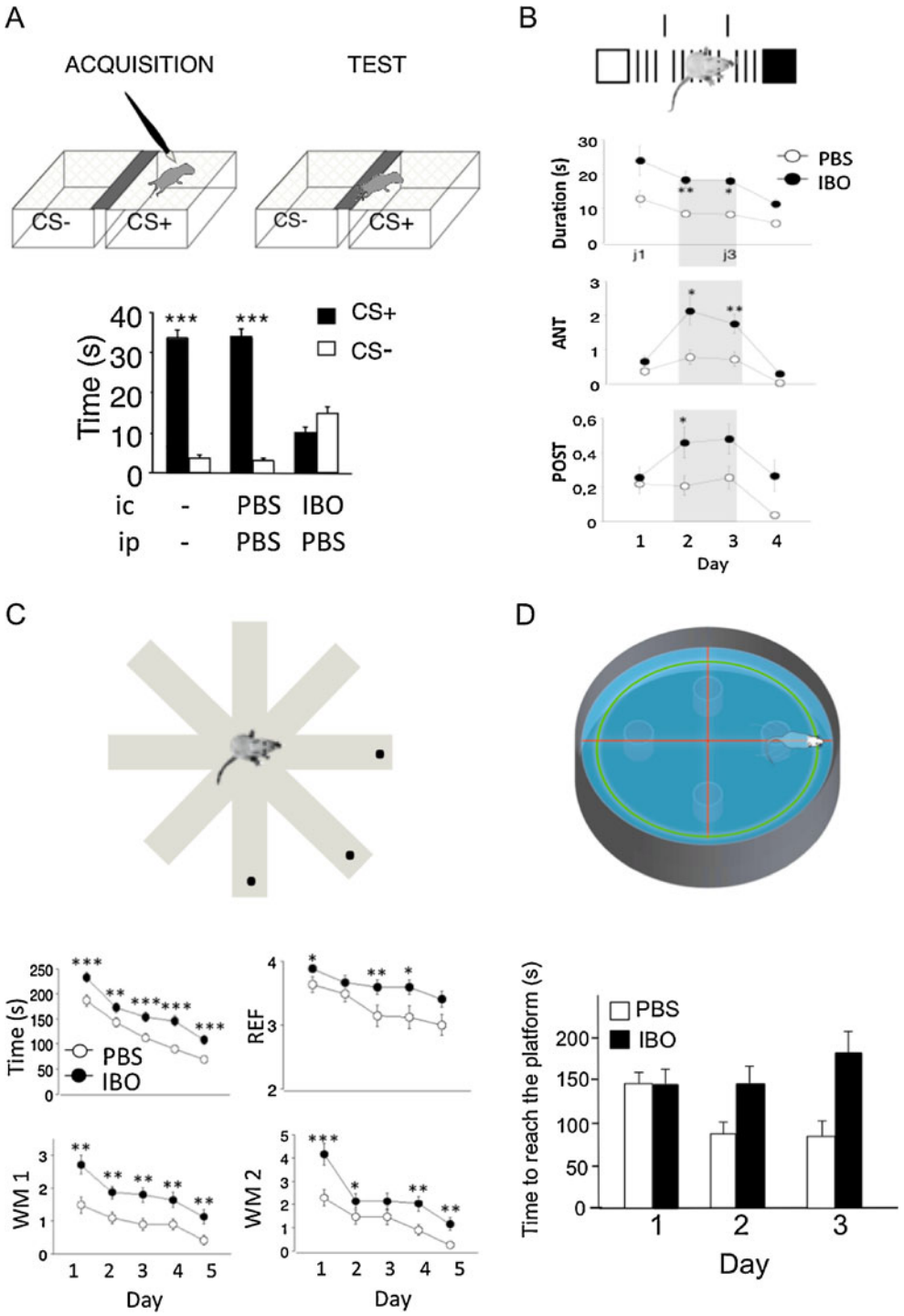
Basic Physiological Functions PhysioPups is a system able to monitor four freely moving pups simultaneously for breathing patterns, heart rate, temperature, ultrasonic vocalizations (indicators of stress and social interactions), and motor activity (Fig. 3). Critically, mouse pups can be monitored from the day of birth to weaning. All functions are measured simultaneously and non-invasively at controlled ambient temperature, and gas environments. Breathing variables (frequency, tidal volume, mean flow rate, and apneas) are measured using whole-body flow barometric plethysmography. Heart rate is recorded using contact surface electrodes incorporated in the floor of the measurement chamber. Body temperature is measured by infrared emissivity of the interscapular region (which contains the heat-generating brown adipose cells). Ultrasonic vocalizations are recorded with a microphone. Gross body movements are calculated from the variations of the respiratory signal baseline. In addition, the physiological status of the newborn mice is analyzed using oxygen consumption (VO_2) measurements in steady conditions and in response to hypoxic challenges, pulse oximetry, and blood gas analysis.

Illustrating the utility of the novel Physiopups platform, we performed a thorough analysis of the basic physiological state of early postnatal pups in response to a moderate inflammatory stimuli [51]. It was identified there were subtle effects of inflammation on breathing pattern (increased apneas), which however were not physiologically significant, thus discarding this possible confounder of pathophysiological mechanisms of brain injuries [51]. These data reveal an important strength given to mouse models by this type of technology, to assess possible covert comorbidities that may cause or aggravate the clinical status of the animal. This technology has also been used to characterize early respiratory disorders of genetic origin [153], and to conduct

Fig. 4 Motor and cognitive deficits in mice with postnatal brain injuries. **A** Disruption of conditioning in newborn mice. Acquisition consisted in placing the pup over the conditioning stimulus (*CS*; i.e., odor) while stroking it with a paintbrush to mimic maternal interaction and then placing the pup over a different conditioning odor without stroking. Effective conditioning was reflected by longer time over the CS with stroking (*CS+*) than over the CS without (*CS−*). Conditioning was completely abolished in the pups with ibotenate-induced lesions [154, 155]. ic, intracerebral and ip, intraperitoneal. **B** Deficits in motor coordination in adult mice. Locomotor abilities were assessed using the Locotronic® device, composed of a horizontal ladder inside a darkened tube. Following twice daily training for 3 days, two rungs of the ladder were removed and animals tested twice daily for two additional days. Coordination was assessed by the number of steps, time to complete, and the number of forelimbs or hindlimb placement errors. The mice with brain lesions showed significantly poorer scores. **C** Deficits in memory in adult mice. Working and reference spatial memory abilities were assessed on an eight-arm radial maze. Animals were habituated to the maze for 5 min a day for 3 days where all arms were contained a baited food cup. During testing (5 days) food was placed into the same four cups at the end of the arms. Each animal was given 5 min to enter each of the baited arms and a working memory error was recorded when a mouse reentered a (now empty) baited arm (*WM1*) or a non-baited arm (*WM2*) during the same trial. A reference memory error (*REF*) was recorded each time a mouse entered an arm that was never baited. The mice with brain lesions showed significantly poorer scores. **D** Impaired spatial learning in adult mice. Spatial learning was assessed using a Morris water maze which consisted of a large circular pool that was filled with water and provided with a circular escape platform 1 cm below the water surface. To locate the platform, the mice relied on visual cues around the maze. Spatial memory was assessed by the time to reach the hidden platform across successive day sessions. Mice with brain lesions did not improve their scores, in contrast with the control mice. Significant differences between groups (ANOVA and Student's *t* test): $*p<0.05$; $**p<0.01$; $***p<0.001$. Values are means±SEM

safety analysis of melatonin [154] or other neuroprotective candidate drugs [155].

Psychomotor Evaluation Infants at 1–3 months of age have movement deficits after preterm birth, which are useful predictors of long-term outcome [156]. Neogait assesses the ontogenesis of gait through image processing that allows automatic detection of contacts of newborn rodent with the floor. A high-resolution video camera observes the pup through the flooring of the apparatus. The images recorded are computed, allowing analysis of overall of parameters such as contact area, stride length, speed and limb bias. The system is composed of an acrylic plate, internally illuminated by infrared and covered by a custom gel, on which the animal moves freely. The system is equipped with heaters allowing full control of the animal environment temperature



and surrounded by an opaque and soundproof plastics barrier to prevent disturbance of the pups that are highly sensitive to changes in light and sound. The Neogait assessment is performed in addition to the Fox test battery which determines developmental milestones based on sensorimotor reflexes in developing mice [146]. This Neogait technique proved efficient to detect early locomotor deficits in newborn mice prenatally exposed to hypoxia (Ringot and Gallego, unpublished data) or newborn rats exposed to postnatal hypoxic–ischemic event (Nguyen and Gallego, unpublished data). Furthermore, in newborn rats exposed to postnatal hyperoxia, early associative abilities as assessed by thermal conditioning were disrupted and inhaled NO treatment maintained learning scores to a level similar to that of control pups [157].

Cognitive Assessment Associative learning is the process by which previously unassociated ideas and experiences are linked together. Associative learning, which includes classical and operant conditioning is a fundamental aspect of memory and learning that underlies many aspects of higher-level cognitive activity. Associate learning has been successfully tested in infants as early as 1 day of age [158], and in newborn mice, we have validated that associative abilities can also be assessed from the first postnatal day onward using a classical conditioning paradigm that pairs novel odors with stroking [151]. This odor-stroke conditioning has been used to show excitotoxic brain lesions were accompanied by early learning deficits [154, 155] (Fig. 4a), long before behavioral defects are detected using conventional tests in adult mice (Fig. 4b–d). This odor-stroke paradigm has also been used to assess the functional efficacy of neuroprotectants [154, 155] (Fig. 5a). In particular, decreases in excitotoxic brain lesion in melatonin-treated neonatal mice correlated with improved early associative learning [154]. In addition, we developed a novel method, thermal-odor conditioning, which takes advantage of the thermotaxic behavior of newborn mice and is less experimenter dependent than odor-stroke conditioning [159]. Neonatal mouse pups preferred the odor that had previously been paired with a warm environment to an odor paired with a cold environment, hence showing conditioning. The conditioning proved effective and reproducible from 5- to 10-day-old pups, and conditioning was established after only three short acquisition trials, indicating a remarkable sensitivity of thermotaxic responses to learning processes.

Monitoring Functional Deficits in the Adult Brain

It is important that early neurobehavioral and neuropathological improvements persist and translate to improved function. We will provide only a short discussion of adult behavioral testing, as the specificity and relevance of these tests has been described in detail elsewhere [145, 160, 161].

Motor Deficits In adulthood, 5 months after perinatal excitotoxic lesion mice presented locomotor coordination impairments (Fig. 4b). Locomotor ambulation assessed using the Locotronic device showed a motor deficiency in lesioned mice, but spontaneous motor ambulation on a smooth even surface was similar for controls and lesioned mice. These findings indicate that the motor deficit observed

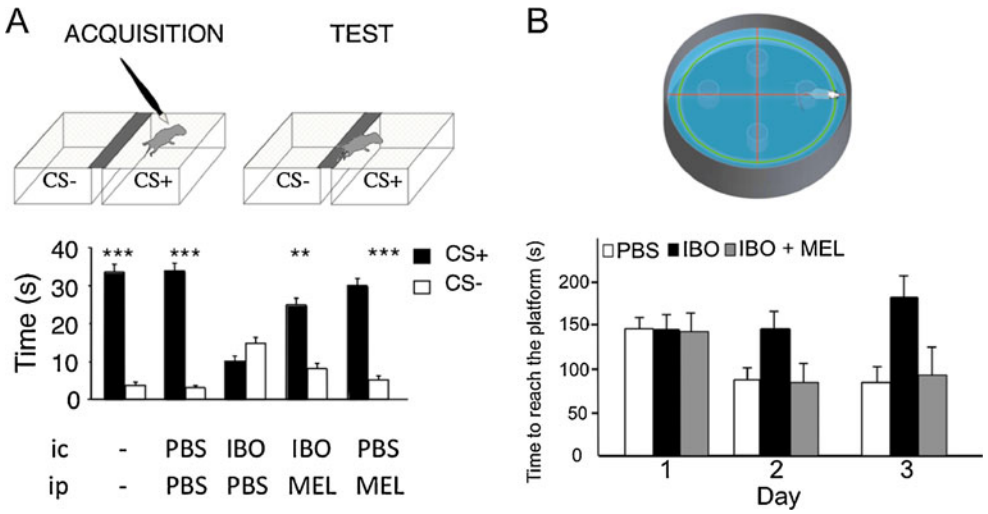


Fig. 5 Prevention of learning deficits by melatonin in mice with postnatal brain injuries. Melatonin (*MEL*) treatment prevented learning abilities in newborn mice with ibotenate-induced injuries. MEL or PBS were given (ip) 15 min before the intracerebral injection (ic) of ibotenate (*IBO*) or PBS. MEL prevented deficits in odor preference learning in newborn mice a, and learning score in the Morris water maze. **b** Refer to Fig. 4 for a detailed description of the tests. Significant differences between groups (ANOVA and Student's *t* test): *$p<0.05$; **$p<0.01$; ***$p<0.001$. Values are means±SEM

in brain-lesioned animals depends on the difficulty of the task. Further evidence is found in the ladder runway task, for the parameter "latency to cross" which was longer for lesioned mice than for controls, lesioned animals making more leg placement errors. The difficulty of the test is twofold: first, the animal's stride has to be long enough to bridge the gaps between the rungs, and there must be efficient coordination between the front and hind limbs and without any visual input. Thus, a battery of adult behavioral assessments may be required to identify deficits in difficult or stressful situations or subtle improvements.

Spatial Memory Spatial memory is a fundamental, cross-species cognitive process, which can be easily operationalized in multiple ways (labyrinths, watermaze, etc.). Spatial memory dysfunction is often reported in children born preterm [162, 163], and assessment of this type of learning forms an important part of experimental adult assessments. Accordingly, in the eight-arm radial maze test (Fig. 4c), adults with a perinatal excitotoxic brain injury had significantly fewer correct responses than the control group, and that poor performance was linked to deficits in both working memory and reference memory (Bouslama and Gallego, unpublished data). Differences in motor function (tests described above) may be a factor influencing scores of spatial memory testing, and as such spontaneous activity parameters must always be considered, but in this study no difference was found between controls and brain-lesioned animals. The difference observed in the memory task therefore appears to indicate a specific memory deficit. The Morris water maze is also used for assessing adult spatial memory and in animals with a perinatal excitotoxic lesion treated with melatonin, in adulthood this test reveals there is improvements in learning and memory (Fig. 5b).

Future of Preclinical Small Animal Behavioral Testing in Neonates

We have outlined above recent advances in small animal testing that will greatly improve the utility of small preclinical animal models. To expand on this, a new set of tests is being developed to provide an even more comprehensive characterization of the safety and the efficacy of any drug targeting the neonate population. These include, (1) the detection of labored breathing in newborns and classification of central and obstructive apneas [164], (2) full quantification of gait development using quantitative markers of crawling, pelvic lift, pivoting, and coordinated gait [165], (3) development of conditioning protocols of classical conditioning for specific purposes (e.g., trace conditioning for short-term memory assessment and reversal conditioning for perseverative behavior assessment) [166], (4) extension of cognitive tests to operant conditioning paradigm [167, 168], and (5) EEG detection of sleep/wake states, sleep states, and seizures.

Conclusions

In this review, we have illustrated an idealized pipeline for testing new neuroprotectants and highlighted new technologies (especially in small animals) that can facilitate the validation of new therapies. Enormous efforts of the research community have provided hypothermia as a viable (but still limited) neurotherapeutic but experimental and preliminary data in humans also indicate the therapeutic potential of melatonin against perinatal brain injury. Even if this suggested potential is proven, the complexity of the human condition means we are likely to need additional neuroprotective and regenerative strategies. However, over the past decade we have acquired many new tools and by embracing this new technology we can expedite the screening of novel therapies. We hope with these tools and an integrated bench to cribside strategic plan this will allow us to fulfill our overarching goal, improving the long-term brain health and quality of life for infants suffering perinatal brain injury.

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