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Review Article

Juvenile Animal Toxicity Studies of Pharmaceuticals and Industrial Chemicals for Regulatory Preclinical Safety Assessment

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ABSTRACT

Juvenile Animal toxicity Studies (JAS) are conducted to evaluate potential safety risks, particularly those affecting postnatal growth and development, that may not be detectable through standard toxicity studies or pediatric clinical trials. These studies play a crucial role in identifying developmental toxicities and supporting the safe inclusion of children in clinical research. Unlike traditional reproductive and developmental toxicity tests, JAS are not guided by rigid regulatory frameworks and are designed on a case-by-case basis. To address this, regulatory agencies such as the ICH, FDA, and EMA have introduced guidelines to support the appropriate planning and execution of these studies. Specifically, ICH M3(R2) and the recently finalized ICH S11 provide harmonized standards for nonclinical safety testing in pediatric drug development, emphasizing flexibility based on the drug's characteristics and target population. Key considerations in designing JAS include the drug's intended use, target pediatric age group, treatment duration, and species-specific differences in pharmacokinetics and toxicity. Special attention is required when the drug may impact developing organ systems like the kidneys, bones, or central nervous system. The relevance and reliability of JAS findings largely depend on appropriate species selection and treatment timing within the developmental window. In the context of regulatory preclinical safety assessment, both pharmaceuticals and industrial chemicals must undergo rigorous toxicological evaluation to ensure safety, particularly for vulnerable populations such as children. For pharmaceuticals, JAS provide critical data for pediatric risk assessment and support clinical trial design. For industrial chemicals, regulatory agencies such as the EPA (Environmental Protection Agency) and ECHA (European Chemicals Agency) may require juvenile toxicity data under programs like REACH (Registration, Evaluation, Authorisation, and Restriction of Chemicals), particularly when there is potential for pediatric exposure. In both sectors, developmental toxicity studies are essential for characterizing age-specific hazards, determining safe exposure levels, and informing labeling and risk mitigation strategies.

Keywords: Pediatric Drug Development (PDD), Juvenile Animal Studies (JAS), Pediatric assessment, Juvenile Animal toxicity studies, Regulatory guidance, ICH S11, ICH M3(R2).

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INTRODUCTION

Overview of Juvenile Animal Toxicity Studies and Regulatory Perspectives

Nonclinical toxicity testing in juvenile animals is a critical component in identifying and characterizing potential safety hazards of chemical and pharmaceutical exposures in pediatric populations, including infants, children, and adolescents. These studies are particularly important for assessing the risks of pediatric formulations, as they consider both direct and delayed effects, such as impacts on growth, sexual maturation, and adverse outcomes that may appear only after exposure has ended (*INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED GUIDELINE NONCLINICAL SAFETY TESTING IN SUPPORT OF DEVELOPMENT OF PAEDIATRIC PHARMACEUTICALS S11 Final Version*, n.d.; Rao *et al.*, 2021).

Juvenile animal studies are more complex than standard adult toxicology studies due to the need to mimic the wide range of human postnatal developmental stages from premature neonates to adolescents (Ayuso *et al.*, 2020). These stages involve the maturation of various organ systems and may include indirect exposures through the placenta or breast milk. The design and interpretation of such studies are therefore more intricate, and

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a number of regulatory frameworks have been developed in response (Brent, 2004; Vrolyk *et al*., 2024).

Historically, aspects of juvenile toxicity testing were embedded within reproductive toxicology guidelines, such as the OECD 416 (Two-Generation Reproduction Toxicity Study) and OECD 443 (Extended One-Generation Reproductive Toxicity Study), which include evaluation of juvenile exposures. Additionally, OECD 424 and 426 provide specific protocols for (developmental) neurotoxicity assessment. In the U.S., the EPA's 870.6200 and 870.6300 guidelines serve a similar purpose for evaluating neurodevelopmental risks (Meshram *et al*., 2023; Oecd, n.d.; *Test No. 443: Extended One-Generation Reproductive Toxicity Study*, 2018).

Legislative efforts such as the FDAMA, Best Pharmaceuticals for Children Act (BPCA), and Pediatric Research Equity Act (PREA) helped establish a regulatory foundation for pediatric drug safety testing. Before their enactment, a large percentage of medications used in children lacked pediatric-specific safety data - over 80% prior to 2012. The permanent reauthorization of these acts has driven improvements in pediatric labeling and safety testing (Best Pharmaceuticals for Children Act and Pediatric Research Equity Act Status Report to Congress, 2015; Califf, 2016; Christensen, 2012a; Hill *et al*., 2013).

Recognizing inconsistencies across global regulatory submissions and the need for a harmonized approach, the ICH convened an S11 Expert Working Group in 2014. This led to the development of the ICH S11 guideline, finalized in April 2020, titled *"Nonclinical Safety Testing in Support of Development of Pediatric Medicines."* This document complements the ICH E11 guideline on pediatric clinical investigation and addresses nonclinical testing strategies for small molecules, biologics, and certain pediatric oncology drugs (in conjunction with ICH S9/S920), but excludes products like vaccines, gene therapies, and tissue-engineered therapies (*INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED GUIDELINE NONCLINICAL SAFETY TESTING IN SUPPORT OF DEVELOPMENT OF PAEDIATRIC PHARMACEUTICALS S11 Final Version*, n.d.).

The ICH S11 guideline emphasizes the need to evaluate not just the active pharmaceutical ingredient but also excipients, considering historical cases of pediatric harm from supposedly inert components. Juvenile animal toxicity studies are used to support a new pediatric specific drug, extention of adult approved drugs for pediatric use and drug already use off-label in children without formal pediatric approval: (*INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED GUIDELINE NONCLINICAL SAFETY TESTING IN SUPPORT OF DEVELOPMENT OF PAEDIATRIC MEDICINES S11*, n.d.; Step, 2020a).

Since many organ systems including the brain, kidneys, lungs, bones, liver, and immune system undergo significant development after birth, careful study design is needed to assess potential toxicity. Different organs develop at different rates and times, so timing and targeting are essential when designing juvenile studies to ensure a thorough safety evaluation (Behrman *et al*., 2007).

This topic was the focus of a symposium session titled "Regulatory Perspectives on Juvenile Animal Toxicologic Pathology", which included expert discussions on these evolving regulatory and scientific approaches and this study looks into the importance and planning of Juvenile Animal Toxicity Studies (JAS) in identifying health risks during development and ensuring the safety of children when assessing drugs and industrial chemicals. JAS are particularly valuable for uncovering age-related toxic effects that might be missed in standard testing or clinical trials involving children, especially when it comes to impacts on growing organs. The research underscores the role of these studies in guiding the safe use of medicines and chemicals in pediatric populations. It also considers current regulatory standards, including ICH S11, ICH M3(R2), and those set by organizations like the FDA, EMA, EPA, and ECHA. These frameworks promote scientifically sound, ethically conducted studies that are tailored to a child's stage of development, often using a flexible approach based on the specific case to produce dependable safety data.

PEDIATRIC DRUG DEVELOPMENT

Pediatric Drug Development: Misinterpretation and Realities

The phrase Pediatric Drug Development (PDD) is often misapplied in scientific literature, industry discourse, and regulatory documentation. While the general public interprets PDD as the process of creating medications for children, institutions like the FDA and EMA use it more narrowly to mean the requirement for separate regulatory approvals and labeling for drugs used in minors (Rose, 2019; Rose and Grant-Kels, 2019).

PDD is deeply intertwined with political, legal, financial, and clinical matters. In Europe, executing a single Pediatric Investigation Plan (PIP) can cost approximately €20 million, with over 1,000 PIPs implemented so far. In the U.S., pediatric patent extensions can offer pharmaceutical companies hundreds of millions of dollars in benefits. Regulatory systems in both regions have established laws and incentives to stimulate pediatric research (*Paediatric Investigation Plans | European Medicines Agency (EMA)*, n.d.).

Despite good intentions, critics argue that this specialized focus may be flawed. They suggest that because children are not biologically distinct from adults, it may not always be necessary to conduct separate efficacy and safety trials for pediatric use.

Thus, decades of regulatory emphasis on pediatric-specific development may not have been fully justified.

The development of modern pharmaceuticals gained momentum during the scientific revolution following the Renaissance. Key regulatory reforms emerged in the wake of drug-related disasters such as the fatal sulfonilamide elixir incident in 1938 and the thalidomide catastrophe in the late 1950s and early 1960s. These tragedies led to a landmark 1962 U.S. law mandating well-controlled clinical trials for drug approval (Ballentine, n.d.).

In the 1950s, serious side effects from antibiotics in premature infants highlighted the urgent need for pediatric safety standards. By the 1960s, the FDA was overseeing drug advertising, and pharmaceutical companies began including pediatric warnings in product labels largely as a legal safeguard. These labels contributed to the view of children as "therapeutic orphans," deprived of access to many modern medications (Lee, n.d.).

To address these issues, the American Academy of Pediatrics (AAP) and FDA worked together to promote pediatric-specific trials, issuing guidelines in 1977 and 1995. In 1997, the U.S. introduced its first pediatric-focused legislation, offering an additional six months of patent protection to companies that conducted pediatric studies. This was followed by the Pediatric Research Equity Act (PREA) in 2003, which gave the FDA authority to require pediatric studies, albeit without offering incentives. These developments influenced the European Union, which introduced its own Paediatric Regulation in 2006 after a decade of policy discussion (Mccune, 2019; *Pediatric Regulatory Initiatives | Request PDF*, n.d.; Rose, 2019; Ward *et al.*, 2017).

Beyond medicine, the global commitment to children's rights was reinforced by initiatives such as the 1959 UN Declaration of the Rights of the Child and the 1989 Convention on the Rights of the Child.

Pediatric Drug Development: Challenges, Misinterpretations, and the Role of Juvenile Animal Studies

In recent decades, there has been increasing focus on Pediatric Drug Development (PDD), though its definition and implementation are frequently misinterpreted. Many people commonly see PDD as simply developing drugs appropriate for children, but regulatory bodies such as the FDA and EMA define it more specifically as the process of obtaining separate regulatory approval and labeling for pediatric use. This difference in understanding highlights the broader political, economic, and

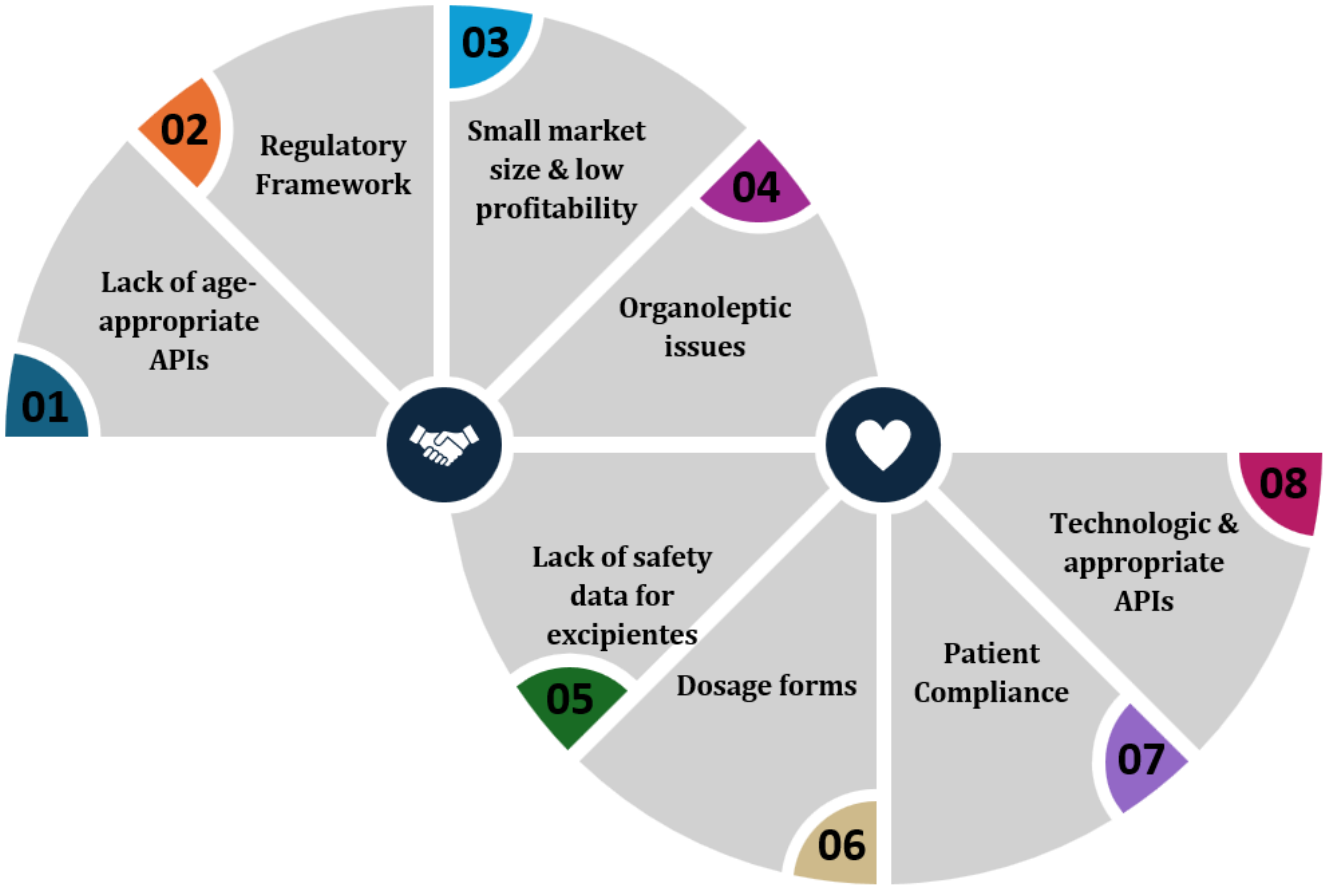

Figure 1: A summary of the factors impacting pharmacotherapy practice and the development of therapeutics aimed at pediatric patients.

legal factors that influence pediatric drug policy (Carleer and Karres, 2011; Spadoni, 2018).

Challenges in pediatric pharmacotherapy and drug development are influenced by multiple factors, as shown in Figure 1.

Reevaluating the Basis of PDD

Several critics contend that the conventional method of conducting separate clinical trials for children is not always scientifically necessary. Given the biological similarities between adults and children, data on safety and efficacy from adult trials can often be reliably applied to pediatric populations when analyzed appropriately. The age categorization of pediatric patients is outlined according to the ICH E11(R1) guideline, as shown in Figure 2. While the initial push for pediatric-specific studies stemmed from concerns about off-label drug use in children, there is increasing apprehension that some age-based regulatory requirements may be overly stringent or unwarranted (*INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED GUIDELINE NONCLINICAL SAFETY TESTING IN SUPPORT OF DEVELOPMENT OF PAEDIATRIC MEDICINES S11*, n.d.; Rose and Grant-Kels, 2019; Ward *et al.*, 2017).

Classification of Pediatric Patients

As summarized, there is considerable heterogeneity in developmental categorization. Central Nervous System (CNS), Blood-Brain Barrier (BBB).

Historical Context and Regulatory Development

Modern drug regulation has its roots in major pharmaceutical crises, notably the fatal sulfanilamide incident in 1938 and the thalidomide tragedy of the late 1950s. These disasters prompted significant regulatory changes, including the introduction of mandatory controlled clinical trials before new drugs could be approved. By the 1990s, organizations like the American Academy of Pediatrics, along with other advocates, successfully promoted policies to encourage pediatric drug research. This resulted in the 1997 U.S. legislation offering extended patent protection for companies conducting pediatric studies, followed by the Pediatric Research Equity Act (PREA) in 2003. Influenced by these U.S. initiatives, the European Union introduced its own Paediatric Regulation in 2006 (Ballentine, n.d.; Mccune, 2019; *Pediatric Regulatory Initiatives | Request PDF*, n.d.).

Juvenile Animal Studies: A Critical Component

Juvenile animal testing plays a crucial role in pediatric drug research, especially when safety concerns cannot be adequately

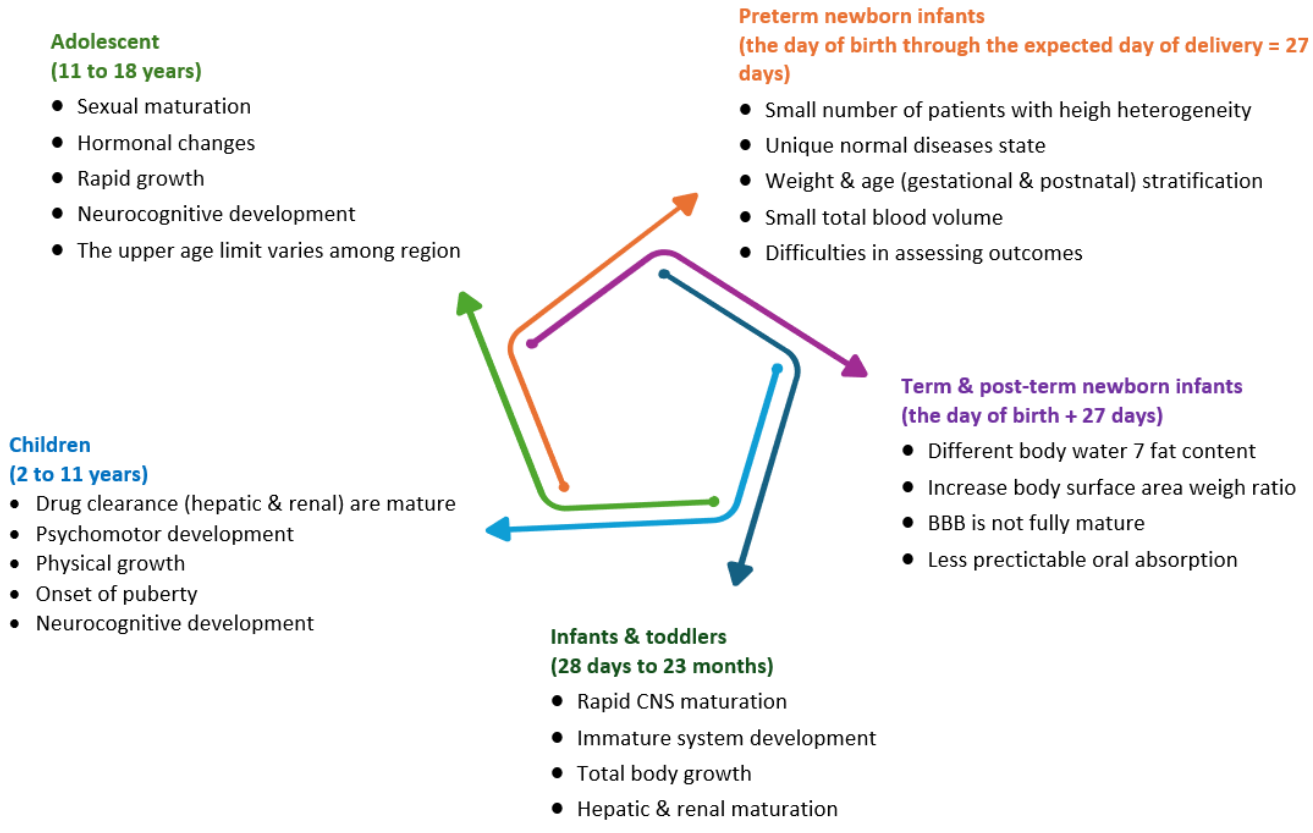

Figure 2: Infographic of the age categorization of pediatric patients according to the International Council for Harmonization (ICH) guideline E11(R1).

evaluated using data from adult studies (Carleer and Karres, 2011). the importance of these studies in identifying toxic effects on immature organ systems and in gaining insights into how drug absorption and metabolism vary between children and adults (Baldrick, 2004). However, these studies present considerable challenges. Careful planning is needed for factors such as species choice, developmental timing, dosing protocols, and study endpoints. While rats are the most commonly used animals, other species like dogs, monkeys, and pigs may be used depending on the drug's target. Differences in growth, organ function, and metabolism make juvenile animal studies complex and occasionally hard to interpret.

CHALLENGES IN PEDIATRIC PHARMACOTHERAPY AND DRUG DEVELOPMENT

International Regulatory Approaches to Juvenile Animal Toxicology Studies

Juvenile Animal Studies (JAS) play a pivotal role in evaluating the safety of drugs and chemicals intended for use in children. These studies are essential for understanding how substances affect immature biological systems, which differ significantly from adult physiology, particularly in how drugs are processed and metabolized. Dr. Alan Hoberman provided a detailed exploration of how regulations governing these studies have evolved, as presented at the Society of Toxicologic Pathology's Annual Symposium (*Pediatric Regulatory Initiatives | Request PDF*, n.d.; Rao *et al*., 2021).

Development of Pediatric Safety Standards

Historical safety concerns, including poisonings from early drug solvents like sulfanilamide and harmful effects from certain drug additives and antibiotics, underscore the long-standing need for pediatric-specific evaluations. Despite these early incidents, global alignment in pediatric testing regulations only materialized in recent years. In 2020, the International Council for Harmonisation (ICH) introduced the S11 guideline, which unified the approach to nonclinical safety studies for pediatric medicines across major regulatory jurisdictions (Ballentine, n.d.; Ich, 2017; *INTERNATIONAL COUNCIL FOR HARMONISATION OF TECHNICAL REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED GUIDELINE NONCLINICAL SAFETY TESTING IN SUPPORT OF DEVELOPMENT OF PAEDIATRIC MEDICINES S11*, n.d.; Mccune, 2019).

Additionally, the ICH E11 guideline highlights the necessity of conducting pediatric-focused drug evaluations and ensuring the development of safe, age-appropriate formulations. This harmonization aligned regulatory efforts across the United States Food and Drug Administration (FDA), the European Medicines Agency (EMA), and Japan's Ministry of Health, Labour and Welfare (MHLW), building on earlier country-specific guidelines issued between 2006 and 2012 (Ich, 2017).

Regulatory Mandates and Compliance

In the U.S., legislation such as the Pediatric Research Equity Act (PREA) and the Best Pharmaceuticals for Children Act (BPCA) require drug developers to prepare Pediatric Study Plans (PSPs), which undergo review by the FDA's Pediatric Review Committee. In parallel, the European Union enforces Pediatric Investigation Plans (PIPs), evaluated by a dedicated Pediatric Committee. These frameworks ensure pediatric considerations are addressed early in the drug development process. Even if a product isn't intended for children, companies must provide justification to obtain a waiver from conducting JAS (Carleer and Karres, 2011; Christensen, 2012b; Mccune, 2019; *Pediatric Regulatory Initiatives | Request PDF*, n.d.; Remick *et al*., 2015; Ren and Zajicek, 2015).

Designing Juvenile Toxicity Studies

Effective JAS design requires careful consideration of the test species, the developmental timing of dosing, treatment duration, and relevance to human pediatric use. These studies typically span developmental windows from gestation (via maternal exposure) through postnatal stages, bridging the gap to adult toxicity testing.

Rodents are frequently chosen due to their well-mapped developmental stages, although other species like rabbits, dogs, or primates may be more suitable depending on the specific drug and pediatric age group involved. Moreover, pediatric drug formulations may contain excipients that differ from adult products, requiring separate safety evaluations (Kim *et al*., 2017).

Behavioral and Neurological Evaluations

Given the ongoing development of the nervous system in juveniles, behavioural testing is a critical component of JAS. The ICH S11 guideline recommends a battery of assessments such as functional observation, reflex testing, motor activity tracking, and memory and learning evaluations. While specific methods are not mandated, all procedures should be validated, appropriate for the species, and aligned with the drug's pharmacological effects. Frequently used tests include: Morris Water Maze to Evaluates spatial memory via hippocampal function, Cincinnati/ Biel Maze to assesses route learning and motor planning linked to striatal circuits, Social Interaction Test Measures sociability, useful in studying effects related to mental health disorders like autism or depression. Each assessment must be carried out by trained professionals following standardized protocols to ensure accuracy and reliability (Step, 2020b).

Linking Behavioural Testing to Brain Structure

To support behavioural findings, detailed anatomical examinations of the central and peripheral nervous systems

are often performed. These neuropathological analyses provide crucial insights into whether the observed behavioural changes have a structural basis, enhancing the overall understanding of a drug's neurodevelopmental impact (Biology, 1989; *The Nervous System and Behavior - Opportunities in Biology - NCBI Bookshelf*, n.d.).

Closing The Gap: Ich S11's Role in Advancing Pediatric Drug Safety Through Juvenile Animal Studies

Pediatric populations have often been overlooked in traditional drug safety evaluations. To address this, the International Council for Harmonisation (ICH) introduced Guideline S11 in 2020 a transformative step toward globally consistent and scientifically grounded nonclinical safety testing for medicines intended for children (Serafini *et al.*, 2024; Tavares *et al.*, 2025).

Addressing Gaps in Pediatric Safety Data

ICH S11 was created to guide the implementation of JAS in cases where existing adult and nonclinical data do not sufficiently ensure drug safety in children. Rather than mandating such studies for all pediatric drugs, the guideline promotes a scientific and risk-based assessment to determine necessity. Its core aim is to improve pediatric safety data while minimizing the use of animal testing.

This balanced approach ensures that studies are conducted only when justified, reflecting both scientific integrity and ethical responsibility (Carleer and Karres, 2011; Mccune, 2019; Serafini *et al.*, 2024).

Core Principles of ICH S11

Scientific Justification: Juvenile animal studies should be undertaken only when clearly warranted. For example, they may be necessary if the target organs are still maturing in pediatric patients, existing data from adults are not predictive of safety in children, drug metabolism and action differ significantly between adults and children (Step, 2020b; Tavares *et al.*, 2025).

Developmentally Relevant Study Design: The guideline outlines best practices for tailoring study designs to match the developmental stages of the intended pediatric age group. Key considerations include choosing animal species that closely resemble human physiology, aligning treatment duration with expected pediatric use and evaluating organ systems essential to development such as the brain, immune system and reproductive organs.

Coordination with Clinical Development: JAS should be timed to support pediatric trials without delaying clinical progress. When adult and existing nonclinical data provide sufficient assurance, early pediatric trials can proceed while deferring JAS until needed.

Animal Welfare and Ethics: Consistent with the 3Rs (Replace, Reduce, Refine), ICH S11 stresses that animal use must be justified, methodically planned, and executed to gain the most value with the least harm.

International Alignment and Benefits

ICH S11 fosters global consistency in how pediatric safety studies are conducted and evaluated, harmonizing expectations across key regions such as the US, EU, and Japan. This harmonization reduces duplication, enhances regulatory efficiency, and supports more ethical practices.

For drug developers, the guideline offers a clear and adaptable framework to generate necessary safety data. For regulators, it provides consistent criteria for evaluating study results. Most importantly, for children, it supports the development of safer, well-tested medications built on strong scientific foundations (Step, 2020b; Tavares *et al.*, 2025).

ADVANCING FUNCTIONAL SAFETY: AN IN-DEPTH REVIEW OF THE M3 R2 GUIDELINES FOR RELIABILITY EVALUATION

Reliability assessment plays a central role in functional safety, particularly for control systems tasked with executing safety-critical operations. The M3 R2 Guidelines provide a systematic and comprehensive approach to evaluating system reliability. This is achieved through the application of the M3 methodology, which integrates statistical failure analysis with architectural system assessment. These guidelines are especially relevant for professionals involved in the design and validation of systems subject to international safety standards such as ISO 13849-1 and IEC 62061 (*INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED TRIPARTITE GUIDELINE GUIDANCE ON NONCLINICAL SAFETY STUDIES FOR THE CONDUCT OF HUMAN CLINICAL TRIALS AND MARKETING AUTHORIZATION FOR PHARMACEUTICALS M3(R2) Current Step 4 Version*, 2009).

Purpose of the Guidelines

The principal objective of M3 R2 is to establish a consistent and transparent framework for evaluating the reliability of complex safety-related systems. It addresses random hardware failures by employing two primary analytical methods: (*INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED TRIPARTITE GUIDELINE GUIDANCE ON NONCLINICAL SAFETY STUDIES FOR THE CONDUCT OF HUMAN CLINICAL TRIALS AND MARKETING AUTHORIZATION FOR PHARMACEUTICALS M3(R2) Current Step 4 Version*, 2009).

Quantitative Analysis

This includes the calculation of reliability indicators such as Mean Time to Dangerous Failure ($MTTF_d$), Diagnostic Coverage (DC), and the assessment of Common Cause Failures (CCF).

Structural Evaluation

Both qualitative and quantitative assessments are performed on the system architecture, taking into consideration subsystem configurations and interdependencies.

Core Reliability Parameters

$MTTF_d$: Represents the expected operational time before a hazardous failure occurs. A higher $MTTF_d$ value corresponds to improved system reliability.

Diagnostic Coverage (DC): Reflects the capability of the system to detect dangerous faults. The guidelines define standardized levels of diagnostic effectiveness and describe methodologies for estimation.

Common Cause Failures (CCF): Pertains to the likelihood of multiple failures arising from a single root cause. The guideline outlines evaluation techniques such as FMEDA (Failure Modes, Effects, and Diagnostic Analysis) and predefined scoring systems to quantify and mitigate CCF risks.

Evaluation Techniques

M3 R2 specifies different levels of assessment depending on system complexity: Basic Component Evaluation Intended for simple or passive components that exhibit minimal failure variation, Complex Subsystem Assessment: Applies to components with integrated diagnostic functions, typically requiring detailed FMEDA-based analysis, System-Level Reliability Analysis: Encompasses a holistic review of Safety-Related Control System Parts (SRP/CS) using advanced modeling techniques such as reliability block diagrams or Markov chains (*INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED TRIPARTITE GUIDELINE GUIDANCE ON NONCLINICAL SAFETY STUDIES FOR THE CONDUCT OF HUMAN CLINICAL TRIALS AND MARKETING AUTHORIZATION FOR PHARMACEUTICALS M3(R2) Current Step 4 Version*, 2009).

Documentation and Compliance Verification

Accurate and thorough documentation is essential for demonstrating compliance. The guidelines require a detailed reliability analysis report, supporting documentation for assumptions and computational data, Justification of failure rates and diagnostic performance, evidence of adherence to system safety and architectural design requirements.

Verification may include design reviews, simulated failure scenarios, and in certain cases fault injection testing to confirm system integrity under failure conditions.

Industrial Relevance

The M3 R2 Guidelines are closely aligned with contemporary machinery safety regulations and support compliance with defined Performance Levels (PL) and Safety Integrity Levels (SIL). These practices are of particular importance in domains such as robotics, automated manufacturing, and other sectors where electronic control systems are integral to safety performance. By adopting the M3 R2 methodology, developers are equipped to design and validate systems that fulfill regulatory obligations while maintaining high standards of reliability and operational safety. This contributes to improved end-user protection, more efficient certification procedures, and increased assurance in the dependability of safety- critical systems (*INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE ICH HARMONISED TRIPARTITE GUIDELINE GUIDANCE ON NONCLINICAL SAFETY STUDIES FOR THE CONDUCT OF HUMAN CLINICAL TRIALS AND MARKETING AUTHORIZATION FOR PHARMACEUTICALS M3(R2) Current Step 4 Version*, 2009).

Neurobiological Considerations In Juvenile Toxicity

Study Design

The increased vulnerability of the developing nervous system to foreign substances (xenobiotics) is a key reason for conducting juvenile toxicity studies. These studies often focus on evaluating neuropathology, driven by both specific neural developmental processes and broader developmental concerns (Grandjean and Landrigan, 2014; Serafini *et al.*, 2024; *The Nervous System and Behavior - Opportunities in Biology - NCBI Bookshelf*, n.d.).

Decisions to initiate such studies typically stem from an understanding of how the Central Nervous System (CNS), particularly the brain, develops over time. The maturation of the vertebrate brain is categorized into stages that correspond with physical and, more recently, molecular or biochemical indicators. While the order of these developmental stages is consistent among species, the timing can vary depending on the species' gestational period and lifespan (Biology, 1989; Bolon *et al.*, 2021; *The Nervous System and Behavior - Opportunities in Biology - NCBI Bookshelf*, n.d.).

During the embryonic phase, the foundational structures of the brain begin to form. Significant brain development including the creation, movement, and specialization of neurons and glial cells takes place from the fetal stage through early childhood. However, different brain areas mature at different rates. The process of forming synapses (synaptogenesis) is active from fetal life through adolescence and continues, to a lesser degree, into

adulthood. Myelin, the protective covering of nerve fibers, begins to form gradually in late pregnancy (third trimester in humans and around the first week after birth in rats) and continues to increase during adolescence (Ackerman, 1992).

Because of this prolonged period of brain development, the CNS remains susceptible to toxic substances throughout both prenatal and postnatal life. Nonetheless, specific regions and cell types within the central and peripheral nervous systems have shorter, well-defined windows of vulnerability. For adolescent humans (12-18 years), additional juvenile animal testing is generally not required, since standard toxicity studies in young adult rodents and non-rodents already cover this developmental phase (Ackerman, 1992).

For drugs intended for very young populations such as preterm and full-term newborns (birth to 27 days), infants (28 days to 23 months), and children (2 to 11 years) juvenile animal studies are often necessary (Figure 2). This is because safety information derived from adult studies may not reliably predict how immature systems, particularly the brain, will respond especially during periods of rapid brain growth (Zisowsky *et al.*, 2010).

Juvenile studies become essential when there is a risk of harm to one or more developing organs, with the nervous system being a frequent concern. Several classes of drugs are known to impact the developing brain. These include alcohol, anesthetic agents (like ketamine and isoflurane), antiepileptic medications (such as phenytoin, sodium valproate, and vigabatrin), chemotherapy drugs, retinoids, stimulants (e.g., caffeine and methamphetamine), environmental pollutants (including chemicals and heavy metals), and even some naturally occurring metabolic byproducts (Ackerman, 1992).

Summary of Neuropathology Evaluation in Juvenile Toxicity Studies

Neuropathology is crucial for assessing neural development in animal toxicity studies. Structural endpoints are particularly useful for developmental screening due to their consistency across

Table 1: Selected Developmental Neuropathology Endpoints for Juvenile Toxicity Testing.

Evaluation approach	Neuropathology endpoints	Rationale for use
Routine screening	Macroscopic observations. Brain weights. Light microscopic evaluation of hematoxyline and eosin (H & E) stained section.	Conventional anatomic pathology endpoints. Applicable to non-clinical safety studies generally.
Expanded Neurohistopathology	Cell component specific histochemical stains. Axons (bielschowsky's silver). Myelin (Luxol Fast Blue [LFB]). Neurons (Cresyl Violet [CV]). Cell type specific Immunohistochemical (IHC) biomarkers. Astrocytes (Glial Fibrillary Acidic Protein [GFAP]). Microglia (Ionized calcium-Binding Adaptor molecule 1 [IBA1]). Neurons (neurotransmitters or enzymes involved in their metabolism). Peripheral Nervous System (PNS) analysis Sensory ganglia dorsal root ganglia and trigeminal ganglia. Nerve analysis hard plastic embedding.	Procedures suitable for examining the extent & nature of any test article related neural responses. Expanded neurohistopathology sometimes requested by regulatory authorities, so sponsor may decide to include one or more of these endpoints up front. Highlight dead or dying neurons. Most useful for detecting acute death (2-4 days after initial exposure). Collection and evaluation suggested for DNTS. Collection and retention in fixative is usual for JAS Preferred for DNTS, but paraffin is acceptable Used rarely for JAS.
Special techniques	Quantitative analysis (e.g., morphometry, stereology). Special microscopy techniques (Fluorescent bioimaging, in situ hybridization, laser capture \ microdissection, magnetic resonance microscopy, etc.,). Ultrastructural evaluation (usually transmission electron microscopy).	Techniques designed to address specific issues. Used only if necessary to answer a given question.

(Abbreviations: DNTS: Developmental Neurotoxicity Study; JAS: Juvenile Animal Study. The special stains for highlighting myelin (LFB) and specific groups of neurons (CV) are often combined on a single section).

laboratories. Both Developmental Neurotoxicity Studies (DNTS) and Juvenile Animal Studies (JAS) include neuropathological evaluations, but their designs differ because of their distinct goals and exposure durations. Various evaluation approaches, neuropathology endpoints, and their rationale in juvenile neurotoxicity studies are outlined, as shown in Table 1.

In DNTS, the primary focus is on the Central Nervous System (CNS) and, sometimes, the Peripheral Nervous System (PNS) for hazard identification. In contrast, JAS includes these systems as part of a broader, more comprehensive assessment. Both study types use common endpoints, such as gross examination, brain weight measurement, and microscopic analysis of H&E-stained tissue sections.

However, DNTS often employs more advanced techniques, including tissue perfusion, specialized fixatives, and a wider range of brain regions and stains, given its exclusive focus on the nervous system. In JAS, expanded neurohistopathology is applied only when necessary and is generally more limited in scope.

Quantitative analysis is a key feature of DNTS but not typically in JAS, for two main reasons: DNTS exposures occur during critical neurodevelopmental stages, making it more likely to detect structural changes. In JAS, particularly when dosing begins later (e.g., postnatal day 22), many neurodevelopmental processes are already completed, making structural analysis less useful. Instead, functional outcomes like behavioral assessments are often more sensitive in detecting effects.

The effects of toxic substances vary depending on the developmental stage at the time of exposure. Early in pregnancy (before gestation day 16 in rats or during the first trimester in humans), exposure often results in embryo death or visible defects, such as neural tube abnormalities or underdeveloped brains (microencephaly). In contrast, later exposure during pregnancy or after birth typically causes more subtle changes, including misplaced or fewer neurons, fewer synapses, impaired myelin formation, and functional issues like behavioral or cognitive deficits.

CONCLUSION

Juvenile Animal toxicity Studies (JAS) and Pediatric Drug Development (PDD) are both key to protecting children from harmful effects that may arise from exposure to drugs and chemicals. JAS are particularly useful in identifying toxic effects on developing organs—issues that may not be detected in adult studies or even in pediatric clinical trials. Regulatory agencies such as the FDA, EMA, and ICH have introduced guidelines to ensure these studies are designed responsibly and reflect the stages of child development. Still, designing and conducting JAS is challenging due to the complexity of mimicking various stages of human growth after birth.

At the same time, the concept of PDD has developed alongside changes in science, law, and policy. Although PDD was introduced to fill the gap in pediatric-specific drug data, some experts now question whether separate clinical trials for children are always necessary. Since many biological functions are similar in children and adults, properly analysed adult data could often be applied to pediatric use. Laws like PREA, BPCA, and the EU's Paediatric Regulation have helped improve pediatric drug labelling, but they've also added significant cost and effort. Moving forward, a balanced approach is needed one that ensures children's safety without placing excessive demands on the drug development process. JAS continue to play a central role in this goal by helping to identify age-related toxicities early on.

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ABBREVIATIONS

JAS: Juvenile Animal Studies; **PREA:** Public Assessment Report; **BPCA:** Best Pharmaceuticals for Children Act; **PDD:** Pediatric Drug Development; **ICH:** International Council for Harmonisation; **EMA:** European Medicines Agency; **FDA:** Food and Drug Administration; **DNTS:** Developmental Neurotoxicity Studies; **FMEDA:** Failure Modes, Effects, and Diagnostic Analysis.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

All juvenile animal toxicity studies mentioned in this article were referred in compliance with national and international standards for the ethical treatment of laboratory animals. These included adherence to the OECD Guidelines for the Testing of Chemicals and the ICH E11(R1) guideline on pediatric medicinal product investigation. When required, studies received approval from Institutional Animal Care and Use Committees (IACUCs) or comparable ethical oversight bodies. The inclusion of juvenile animals was scientifically warranted to evaluate developmental and safety risks that cannot be adequately studied in adult models. Every effort was made to apply the 3Rs principles Replacement, Reduction, and Refinement to ensure responsible and humane use of animals.

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