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Juvenile Toxicity Testing


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Regulatory status of juvenile technology

US – Food and Drug Administration (**FDA**)
Guidance document: Nonclinical Sa1fety Evaluation of Pediatric Drug Products (**2006**)

Europe – European Medicines Agency (**EMA**)
Guideline on the need for Nonclinical Testing in Juvenile Animals on Human Pharmaceuticals for Pediatric Indications (**2008**)

Japan – Ministry of Health, Labour and Welfare (**MHLW**)
Guideline on the Nonclinical Safety Study in Juvenile Animals for Pediatric Drugs (**2012**)

ICH – M3(R2): If primary use is pediatric a modified chronic study starting dosing at juvenile ages can be conducted

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Regulatory status of juvenile technology

ICH S-11 – *Guidance in progress* – Finalized in **2020** and will harmonize these documents


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Species selection

Rodents and non-rodents

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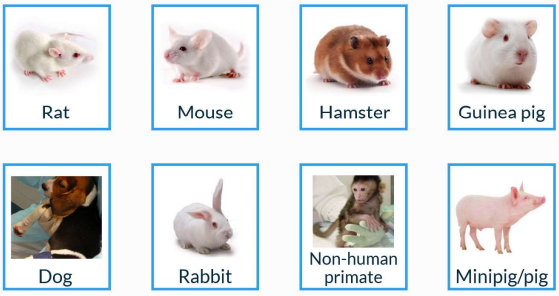
Juvenile toxicology species



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Desirable characteristics

Rodents	Nonrodents
Rats Estrous cycle, reproductive capacity, litter size, short gestation, skeletal growth, immunological assessments, and neurobehavioral assessments	**Non-human primate** Several anatomical and maturation characteristics similar to humans, including use of human pediatric screens to evaluate neurobehavior
	Minipigs Several anatomical and maturation characteristics similar to humans
Mice Estrous cycle, reproductive capacity, litter size, short gestation, immunological assessments, and neurobehavioral assessments	**Rabbit** Reproductive capacity and ocular
	Dog Skeletal growth, pulmonary function, cardiovascular function, renal function, and ocular

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Factors to consider during species selection

Route of administration	Route of blood collection	Technical expertise	Age at weaning/ growth/ maturation
Reproductive capacity evaluations	Litter size	Blood volume	Historical control data
Species availability	Use in adult toxicity	Organ system development (toxicological target organs)	Feasibility of pre/post-weaning evaluations

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Species comparison

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Factor	Rat/Mouse	Rabbits	Minipigs	Dogs	Nonhuman Primate
Animal availability	Routine	Available, but advance notification highly recommended	Available, but advance notification highly recommended	Routine	Can be a problem
Pre-weaning procedures	Routine	Limited	Routine	Routine	Can be (but not typically) done
Age of sexual maturity	9 to 13 weeks	4 to 7 months (strain dependent)	5 to 7 months	12 months (males) and 8 to 10 months (females)	4 to 6 years
Group sizes	10 to 20/sex (depending on endpoints)	10/sex (main) 5/sex (recovery)	6/sex (main) 3/sex (recovery)	5/sex (main) 5/sex (recovery)	5 to 10 juveniles

Dog *vs.* Minipig

Comparison of endpoint examples

Dog	Minipig
• Ophthalmology from PND 21	• Ophthalmology from PND 7

• Blood sampling for clinical pathology
  • From PND 4 jugular vein unanesthetized animal
	• Blood sampling for clinical pathology
	  • PND 14 (hematology only)
	  • PND 21 jugular vein unanesthetized animal

• Cardiovascular examinations
  • From PND 7 (ECG)
  • From PND 10 (ECG and BP indirect)
	• Cardiovascular examinations
	  • From PND 5 (ECG)

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The minipig as an alternative model

Advantages

- Easy to select vigorous **healthy** pups to the study and perform technical procedures/physical examinations etc. as of **PND 1**
- The **developmental periods** are relatively **short enough** to consider **long-term studies** (including reversibility)
- **Physical size** during the minipig developmental "windows" is very conducive for technical procedures/assessments

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The minipig as an alternative model

Advantages

- **Cost** is **comparable** (potentially less) with juvenile dog model?
- Cyclicity feasible for reproduction testing
- Immunology assessments

The minipig as an alternative model

Disadvantages

- **Limited historical control data**/references (publication in preparation)
- **Venous access** gets **difficult** for blood sampling and IV dosing as the animals get older
- **Skeletal growth** continues post puberty for a **longer time**
- **Advanced growth rate** and **maturity at birth** (*e.g.*, neuromuscular and respiratory systems) may lead to other animal models being more comparable with the human for a number of compounds

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Study design considerations

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Study design questions

Children are not just little adults

- Indication/age/route/duration of treatment
- Selection of the most appropriate test species: is the rodent acceptable?
- How often will the drug be administered in the clinic?
- How will the drug be administered? Is the same route feasible in animals?

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Study design questions

Children are not just little adults

- Are there any target organs based on adult animal or human data?
- What is the clinical mode-of-action? Is the drug CNS-active?
- Is a recovery assessment warranted based on adult animal or human data?
- Juvenile-only indications? Are 2 species needed?

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When to dose

Age groups	Human	Rat/ mouse (days)	Rabbit (weeks)	NHP (Cyno) (months)	Minipig (days)	Dog (days)
Premature	Less than term	1 to 4	0 to 1-2	-	-	1 to 4/10
Neonate	Birth to 1 month	4 to 7/14	2 to 3	Birth to 0.5	0 to 14	5/11 to 21
Infant	1 month to 2 years	7/14 to 21	3 to 5	0.5 to 5	15 to 28	22 to 42
Children	2 to 12 years	21 to 28F/35M	5 to 13	6 to 35	29 to 108	43 to 180
Juvenile	12 to 16 years	28F/35M to 49F/70M	13 to 21	36 to 48	120 to 180	180 to 270

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Study designs

Species selection | Age(s) of species | Possible approaches | Litter effect

Pharmacodynamics
Toxicological target organ(s) in humans/ potential species
Species selected for adult toxicity studies (already performed)

General - age of weaning
Specific organ system development/overall development (pharmacological and toxicological target organs)
Physical characteristics (age of eye opening)

Split-litter (within litter)
Whole-litter (between litter)
Cross-fostering / fostering (rodents)

Pre-weaning
Post-weaning

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Species-specific technical considerations

Rats *vs.* mice

Rats

- Small molecules are generally not species specific and therefore can be tested in a standard DART animal model
- Well-established experimental designs and well-established historical control data
- Short gestation periods
- Large litter sizes
- Good fertility

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Species-specific technical considerations

Rats *vs.* mice

Mice

- Variable litter sizes
- Small animal, small available blood volume
- Not all strains recommended for reproductive evaluations
  - *E.g.* CD-1 nude mice have problems with ovulation and are unable to lactate
  - Dominant female will suppress ovulation
  - Once mated males are aggressive and cannot be returned to group housing

Technical considerations

Non-routine species

Average pup numbers/litter	
Mouse Strain	**Litter size**
Crl: CD1	10 to 12 pups
Crl: CD1 nude	8 to 10 pups
Crl: C57BL/6	6 to 8 pups
Crl: Balb/c	5 to 7 pups

- Litter size and viability of some strains

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Technical considerations

Non-routine species

Age (post partum day)	Collection Site	Volume Obtained per Pup
7	Cardiac puncture	100 uL
13	Vena cava	100 uL
21	Jugular vein	<200 uL
21	Abdominal aorta	400 – 500 uL
28	Jugular vein	100 uL
35	Jugular vein	150 uL
42	Abdominal aorta	0.8 – 1.2 mL

- Small size of the animal
- Dosing
- Blood collection
  - When do samples need to be obtained?
  - What volume is required for analysis?
  - Which sampling procedure is feasible at this age?
  - Which one will provide the volumes needed for the assay with less difficulty?

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Guinea pigs *vs.* hamsters

Guinea pigs

- Time-mated sows received on GD 32-37
- Long gestation period
- Group housing facilitates optimal nursing and maternal care conditions
- Socialization
- Small litters: <4 (can be up to 8)
- Sows nurse for 3 to 5 days post delivery
- Weaned between days 5 and 10 postpartum and can be co-housed per sex up to 5 weeks of age
- Vitamin C requirement

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Guinea pigs *vs.* hamsters

Hamsters

- Careful consideration must be taken when handling the dams as they are known to cannibalize their litter when disturbed
- Remove the dam away from the litter to a secondary home cage that the dam is exposed to daily prior to delivery, but not the litter from the dam
- Special husbandry techniques required
  - *E.g.*, nesting material should remain undisturbed
  - Frequent glove changes

Nonhuman primates vs. rodents

Parameter	Nonhuman primate	Rodents
Dosing	Standard routes	Standard routes
Housing	Social needs: Two to three per gender in cage	• Pre-weaning: Co-housed with dam and litter • Post-weaning: Individually housed
Feeding	• Amount: 3-4% of BW • Small portions, 3x per day	• Pre-weaning: Nursing and presumed to consume maternal feed after PND 14 • Post-Weaning: Provide fixed amount ad libitum
Handling	• Thin gloves • Thorax, not limbs • Fleece pad for comfort on separation from cage mates	• Fragile • Special precautions needed during IV dosing
Data collection	• Blood volume limitations (only ~1.5 kg at 12 mo old) • Hand-held for ECG	• Terminal blood collections • Blood volume limitations (early life) • Reproductive capacity can be evaluated

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Practical study design considerations

Duration of treatment

- How often is the drug administered in the clinic? Acute? Or chronic?
- ICH M3 (R2) states "*the duration of (treatment) will depend on the toxicity to be addressed, the organ system involved, and the information available from previous studies*"
- Generally accepted range is earliest required to sexual maturation (PND 49 or 70)

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Practical study design considerations

Dose levels

- What determines the high dose? Frank toxicity can cause 2° effects on growth
- The highest no-effect level in adults should generally be selected as high dose
- If there is no effect in juveniles, adult safety margins may be applied (assuming comparable blood levels (exposure)

Practical study design considerations

Duration of recovery period

- What determines the inclusion of a recovery period?
- When included, how long should the recovery period be? Generally 28 days is ok
- What assessments are included?

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Practical study design considerations

Species selection, single species or two species

- ICH M3 (R2) states "*when a juvenile animal toxicity study is warranted, one relevant species (preferably rodents) is generally considered adequate*"
- Juvenile animal studies in **2 species** are rarely recommended
  - Lack of adult human data (*i.e.*, a pediatric-only indication)
  - Multiple issues of developmental concern – and a single species cannot address

Practical study design considerations

Within-litter design

- All groups represented within each litter; accounts for litter effects
- Genotypically similar pups assigned to control and treated groups
- Greater chance of **cross-contamination** (across pups, and also the dam)

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Practical study design considerations

Between litter design

- Entire litter assigned to the same group; makes for a large study
- Not compatible with the 3Rs as a lot of pups within litters are wasted
- Simplification of dosing – all pups in the litter get the same dose
- Reduced chance of cross-contamination

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Cross fostering in rodents

Why is it important?

- Genetic diversity of the pups receiving the test article needs to be accounted for; pups from one litter should never be over-represented within any dose group

	Option 1
Treatment	Treat one/sex/litter at a specific dose of test article
Advantage	Optimal for genetic diversity
Disadvantage	Does not align with 3Rs; up to 720 pups could be wasted based on a main/recovery size of 10/sex/group (does not include special assessments)

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Cross fostering in rodents

Why is it important?

- Genetic diversity of the pups receiving the test article needs to be accounted for; pups from one litter should never be over-represented within any dose group

	Option 2
Treatment	All of the pups in a litter dosed with the same dose level
Advantage	Minimize number of pups
Disadvantage	Insufficient genetic diversity

Cross fostering in rodents

Why is it important?

- Genetic diversity of the pups receiving the test article needs to be accounted for; pups from one litter should never be over-represented within any dose group

	Option 3
Treatment	Treat one/sex within each litter at one of the four doses being used in the study
Advantage	
Disadvantage	Potential cross-contamination from feces and urine, having animals from multiple dose groups within a litter

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Cross fostering in rodents

Why is it important?

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	Option 4
Treatment	Reassign pups from multiple litters to a dam
Advantage	Cross-fostered by breeding facility; Optimal for the number of litters and dams required and at the same time, addresses the issue of dose cross-contamination
Disadvantage	

Standard endpoints

- Detailed clinical observations and survival
- Body weights and food consumption (assessed PND 21 onwards)
- Clinical pathology, organ weights and histology
- Developmental landmarks & landmarks of sexual maturation

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Standard endpoints

- Developmental landmarks & landmarks of sexual maturation
  - Anogenital distance (assessed on PND 1) – rarely included per dosing period.
  - Nipple retention (assessed in males only, on PND 13)
  - Vaginal patency (assessed PND 25 onwards in rat)
  - Balanopreputial separation (assessed PND 35 onwards in rat)
  - Other parameters (eye opening, pinna detachment, surface and air righting reflex, pupillary response etc.)

The unique things about juvenile toxicology studies from adult studies are sexual maturation and normal growth

HSTalks

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Routine evaluations

Earliest day post partum

Parameters	Species					
	Rat	Mouse	Rabbit	Dog	Cyno	Minipig
In-life (clinical signs, body weight)	1	1	5	1	1	1
Food consumption	22	22	28	42	180	28
Clinical pathology	1	1	5	1	14	14
Ophthalmology	21	21	21	21	14	7
Toxicokinetic sampling	1	1	1	1	14	1
Organ wt, gross & microscopic observations	1	1	5	1	1	1

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Dose administration techniques

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Routine evaluations

Age groups	Human	Rat/ mouse (days)	Rabbit (weeks)	NHP (Cyno) (months)	Minipig (days)	Dog (days)
Premature	less than term	1 to 4	0 to 1-2	-	-	1 to 4/10
Neonate	birth to 1 month	4 to 7/14	2 to 3	birth to 0.5	0 to 14	5/11 to 21
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Routes of administration – earliest age

Earliest day post partum

Dose route	Species					
	Rat	Mouse	Rabbit	Dog	Minipig	NHP*
Oral gavage	1	7	4	1 (caps. 42)	1	>2 weeks
Intravenous bolus	4 Intermit-tent	10	14	1	4	>2 weeks
Intravenous infusion	>21	?	?	49 to 56	28(4)?	>2 weeks
Inhalation	4 to 7	21	?(10)	10	?	?
Parenteral (IM/SC)	1	1	4	1	1	>2 weeks
Dermal	10	21	35	42	1	>2 weeks

Gradually improving ages, frequency and volumes
* NHPs younger than 9 months old need to be co-housed with mothers ? – Unknown

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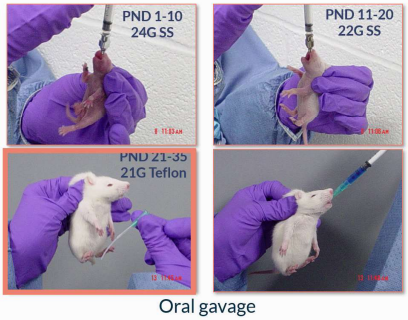
Dosing technique varies for each age



Oral gavage

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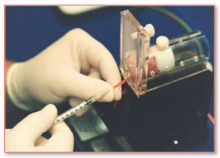
Dosing technique varies for each age



PND 4, intravenous bolus

PND 4, inhalation

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New routes of administration

Mice

- Temporal vein
- Vein anterior of the ear bud that feeds into the jugular vein
- PND 1
- Limited volumes
- Intracerebroventricular injections
- Well-tolerated method to deliver cells, drugs, or viral vectors to the CNS (left or right ventricles)
- Volume limited to 5 mcL per injection
- PND 1 or 2

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New routes of administration

Rat

- Intravenous infusion
- Non-cannulated
- Up to 30 minutes
- PND 21/22
- Subcutaneous infusion via osmotic minipumps
- PND 14 to 42

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Routes of administration - rabbits

Demonstration of oral gavage dosing in a pre-weanling rabbit kit

Demonstration of inhalation dosing in a pre-weanling rabbit kit (PND 15)

	Oral	Intra-peritoneal	Sub-cutaneous	Intra-muscular	Dermal	Inhalation
Dose Volume	1 to 10 mL/kg	1 to 10 mL/kg	1 to 10 mL/kg	0.5 mL/kg	1 to 2 mL/kg	Duration 3 hours
Age at Initiation	PND 5	PND 4	PND 4	PND 4	PND 35	PND 6 (15 min intervals)

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Exposure and microsampling

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Importance of metabolism/toxicokinetics

- Neonate to juvenile to adult, often dramatic differences
  - Human neonates **1/5** of adult to **four to six times** adult rates in children
- Nonclinical changes with age
  - Between **days 7 to 28** post-partum differences in AUC up to **300-fold** have been seen
- Sample blood/plasma/serum all ages
  - Micro-sampling from various sites at different ages
- Sample tissue all ages
- Induction studies

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Juvenile studies and exposure assessment

- Juvenile studies are not useful for predicting exposure as we have limited ability to correlate species-specific timing for maturation of drug metabolizing enzymes
- Bioavailability and biotransformation may be difficult to predict accurately in juveniles based on adult data

Factor influencing exposures	Juvenile animals vs. adults
Gastric pH	Higher
Gastric motility	Lower
Protein binding	Lower
Body fat composition	Lower
Body water and fluids	Lower
Drug receptor expression and binding	Variable
Drug metabolism	Developmentally lagging

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Ontogeny of metabolic capability

Metabolic Parameter	Age of Development		References
	Human	Rat	
Phase I enzymes	Reach adult level at **3-6 months**	Develop **postnatally**	Ginsburg *et al*, 2002; Klinger *et al*, 1981
Carboxyesterases	Reach adult level by **2 years**	Increase during post-weaning **(PND 21-35)**	Bell & Echobichon, 1975; Echobichon & Stephens, 1973; Augustinsson & Barr, 1963
Phase II enzymes	Reach adult level at **3-6 months**	Vary depending upon enzymes – reach adult levels at **puberty**	Ginsburg *et al*, 2002; Lucier, 1981

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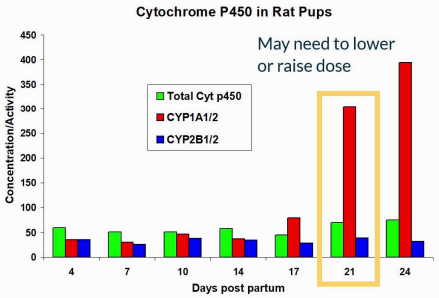
Metabolism/toxicokinetics



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Microsampling

Typical microsamples:

- Biofluid (plasma or serum)
- Approx. **32** to **64 µL** blood into microtubes or capillaries

Possibilities:

- Non-terminal repeated (weekly) sampling from non-anaesthetized pups during the pre-weaning period

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Microsampling – toxicology practicalities

Use fewer rodents in satellite groups	Possibility to exclude satellite animals?	Smaller volume, more samples, animal welfare considerations
Single TK profiles for rodents as standard	Analysis of other biomarkers (main group or satellites)	Regulatory and industry acceptance

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Microsampling – rodent pups

Up to PND 14

Terminal blood samples collected by cardiac puncture or vena cava

Non-terminal techniques sampling from sub-mandibular or lateral tail vein

Using microsampling techniques, one pup can be used per time point

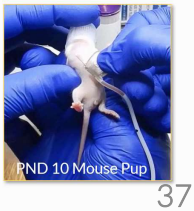
Using macrosampling techniques, pooled blood samples required to obtain sufficient volume for analysis

PND 14 to PND 28

Microsampling from jugular or tail vein allows for serial sampling (up to 2 time points)

≥ PND 28

Microsampling from jugular or tail vein allows for serial sampling (up to 5 time points)



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Microsampling – PND1 rat pup

Blood drawn into hematocrit K2EDTA capillaries following puncture of the jugular vein
60 µL capillary (either 30 or 60 µL possible) and 25G needle

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Microsampling – juvenile rat study design

TK sampling regimen:

Two occasions – first and last day of dosing
Control: 1 time points / collection day
Treated: 6 time points / collection day

Standard sampling regimen:

3M+3F/time point (bleeding each animal twice)
Based on a macrosample of 0.3-0.5 mL

Microsampling regimen:

All animals/time point
Based on a 32 µL blood sample

	Control	Low	Inter	High
Main Study	10M + 10F	10M + 10F	10M + 10F	10M + 10F
Satellite Study: Standard TK Sampling	6M + 6F	27M + 27F	27M + 27F	27M + 27F
Total number of satellite animals = 174				
MICROSAMPLING				
Satellite Study	3M+3F	3M+3F	3M+3F	3M+3F
Total number satellite animals = 24 (86% reduction)				

Alternative: no satellites; microsample main study animals

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Growth and bone assessments

Use of DXA and pQCT

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Growth and physical development

Parameters

- Body weight
- Physical development (rats, mice and dogs [mini-pigs, rabbits])
  - Tooth eruption, eye opening, pinna detachment
  - Vaginal opening, preputial separation, testes descent
  - Anogenital distance (rodents)

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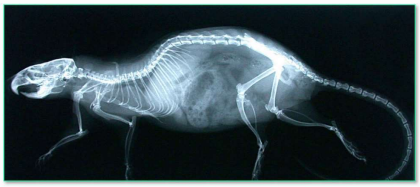
Growth and physical development

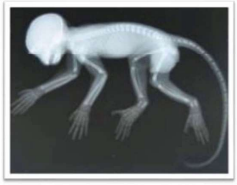
- Skeleton
  - *In vivo* measurements
  - *Ex vivo* measurements and evaluations



Routine assessments of skeletal growth

In vivo physical measurements

- Crown-rump length, tail length, tibia measurements (rodents)
- Crown-rump, external tibia (rabbit)
- Height, length (minipig, dog)
- External measurements, head circumference, external tibia, anogenital distance (non-human primates)



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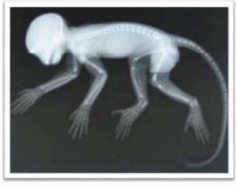
Routine assessments of skeletal growth

In vivo radiographs

- Lumbar spine, femur, tibia

Ex vivo measurements

- Femur and tibia - length and width



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Advanced skeleton assessments

Bone quality	Bone growth dynamics

- Bone mineral content (BMC)
- Bone mineral density (BMD)
- Measure BMC and BMD *in vivo*
- Dual-energy X-ray (DXA) absorptiometry
- Peripheral quantitative computed tomography (pQCT)
- Biomechanical strength testing
- Architecture (histomorphometry)

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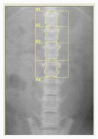
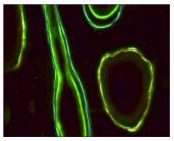
Advanced skeleton assessments

Bone quality	Bone growth dynamics

- Histomorphometry (flurochrome labeling)
- Bone formation rates (BFR), mineral apposition rates (MAR)
- Biochemical markers of bone turnover



DXA scan Rabbit lumbar spine



Calcein green labeling

X-rays

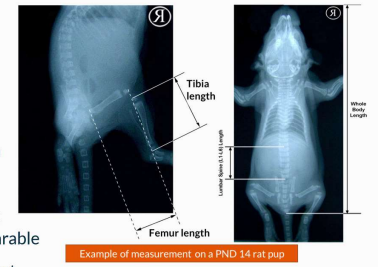
In vivo or *ex vivo* measurements

- Body weight
- Crown to rump
- Bone length and width

Physical or radiographic

Physical and radiographic measurements are comparable

X-rays provide a permanent and verifiable record



Example of measurement on a PND 14 rat pup

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HSTalks

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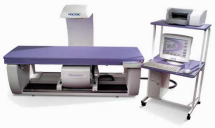
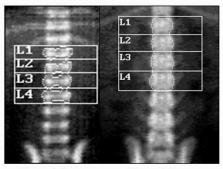
Dual energy X-ray absorptiometry: DXA

- Scan large areas of bone
- BMC converted as areal (2D) BMD
- Body composition for Fat and Lean mass analysis
- 2D densitometry
- "Gold" standard for osteoporosis diagnostic
- Need anesthesia





Day 19 pp Day 63 pp
Lumbar Spine in Juvenile Dogs

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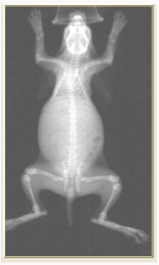
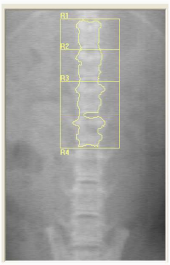
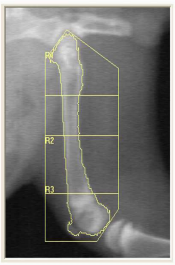
DXA scanning juvenile rabbits



Whole body



Lumbar spine



Femur

Source: Charles River Laboratories, Inc.

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DXA scanning juvenile rabbits

Metaphysis

Increases in area and BMC consistent with growth

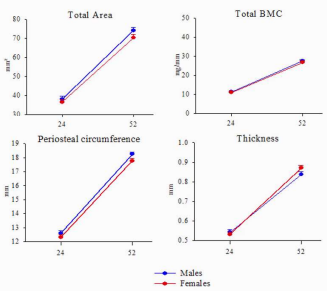
Variability in BMD related to growth

Diaphysis

Increases in bone size (diameter) with age

Associated with increases in cortical thickness

Note: Rabbits reach skeletal maturity shortly after they are sexually mature



Group Mean (S.E.M.) Bone Densitometry Values by pQCT

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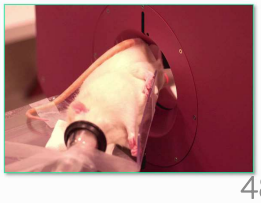
pQCT

- Volumetric BMD
- Differentiation between trabecular and cortical bone
- Single slice through the bone
- Cross-sectional moment of inertia (CSMI) is used as a bone strength parameter



Source: Charles River Laboratories, Inc.

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pQCT

- Volumetric BMD
- Differentiation between trabecular and cortical bone
- Single slice through the bone
- Cross-sectional moment of inertia (CSMI) is used as a bone strength parameter
- Bone mass and its distribution in the bone used to derive surrogate measures of bone strength
- Geometric parameters: periosteal and endosteal circumferences, trabecular and cortical bone areas
- Determine fat and muscle mass with ratios to bone mass

Behavioral assessments

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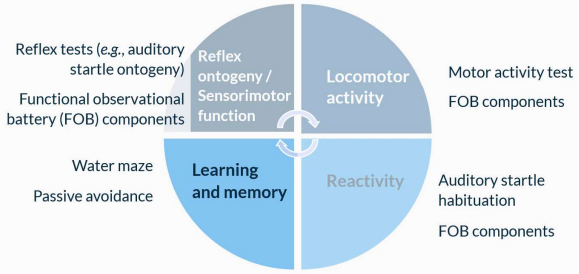
Neurotoxicology – rodents

FDA/EMA requirements for CNS function



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Types of neurobehavioral assessments

Validated at Charles River (various sites, North America and Europe)

	Rat	Rabbit	Dog	Nonhuman Primate
Detailed clinical observations	✓ (Birth)	✓ (≥ PND 4)	✓	✓
Clinical neurobehavioral assessment	–	–	✓ (≥ Day 28)	✓ (≥ Day 7)
Functional Observational Battery	✓ (≥ PND 4)*	✓ (≥ PND 42)	✓	✓
Locomotor Activity	✓ (≥ PND 21)	–	–	–
Auditory startle response	✓ (≥ PND 21)	–	–	–
Learning and memory (water maze or eye blink)	✓ (≥ PND 21) Water Maze**	✓ (≥ PND 110) Eye Blink	–	–
Active/passive avoidance	✓ (≥ PND 21)	–	–	–
Social interactions	✓ (~PND 35)	–	–	–
Nerve conductivity	–	–	✓ (3-4 mon)	

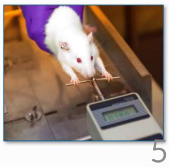
* Some endpoints cannot be assessed in neonatal animals and may be omitted from an FOB assessment; ** Morris, Cincinnati or Biel Maze

Functional observational battery

A temporal assessment of functional status of animals

- Animals are observed and scored on the following sets of criteria:
  - Home cage observations
  - Handling observations
  - Open field observations
  - Sensory & physiological observations
  - Neuromuscular observations

- Inter-observer reliability assessment
- Age of animals
- Sample size



Source: Charles River Laboratories, Inc.

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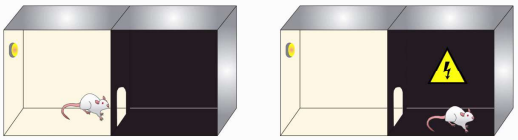
Avoidance testing

Passive avoidance



Rat is placed in the brightly-lit side of the chamber, which is the more adverse side and when it moves into the dark (more preferred) chamber, a foot shock is administered in which the rat quickly learns to remain on the light side of the chamber

Original Design by Ellen McGlinchey

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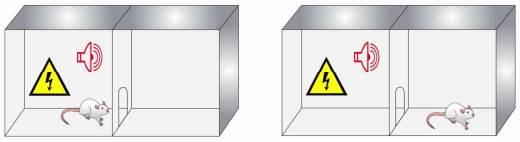
Avoidance testing

Active avoidance



Rat is placed in one side of a chamber where an adverse stimulus (light or tone) is elicited shortly followed by a footnote. The rat then quickly learns to associate the tone with the forthcoming shock, and will actively avoid the shock by moving to the other chamber

Original Design by Ellen McGlinchey

Auditory startle response

Assessment of sensory, motor function and reactivity

- Auditory startle testing is a **non-associative learning** task conducted in a **sound-attenuated room** in specially designed enclosures to measure animal response

Source: Charles River Laboratories, Inc.

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Auditory startle response

Assessment of sensory, motor function and reactivity

- Auditory startle testing is a **non-associative learning** task conducted in a **sound-attenuated room** in specially designed enclosures to measure animal response
- Each session consists of a **5 min acclimation period**, followed by **50 auditory trials**, each with an **8s intertrial interval**
- Each trial consists of a **115 ± 5dB mixed frequency** sound burst stimulus (**~20ms duration**). Responses are recorded during the first **100ms** following onset of the startle stimulus for each trial
- Data are analyzed in **5 blocks of 10 trials** each and peak response amplitude (PEAK) and latency to peak response (T_{peak}) in milliseconds are reported

Locomotor activity assessments

Assessment of the movement of an animal in a novel environment

- Locomotor activity assessments are also conducted in a sound-attenuated room in specially designed enclosures fitted with **photobeams**
- **Exploratory**, **ambulatory** and **grooming behavior** of the animal causes photobeams to be broken, and the number of beam breaks is documented
- Each test session is **60 min** in duration and data are analyzed in **6 blocks of 10 min each**, and over the entire 60 min session
- Parameters evaluated:
  - Total counts
  - Ambulatory activity counts

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Source: Charles River Laboratories, Inc.

Learning and memory assessments

Assessment of an animal's ability to learn, execute and remember a complex task

Types of Learning & Memory Tests

- Classical or Respondent Conditioning Tests
  - Eye Blink Conditioning
  - Pavlov's Dog
- Operant Conditioning Tests
  - Avoidance (Light/Dark Shuttle box with aversive stimulus of electrical shock
  - Water maze, with the averse stimulus of swimming in water

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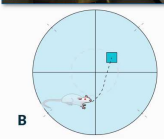
Learning and memory assessments

Assessment of an animal's ability to learn, execute and remember a complex task

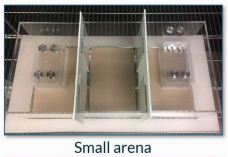
- Morris Water Maze
  (circular pool with submerged platform in different quadrants)
- Cincinnati or Biel Water Maze
  (T-maze with a series of L or R directional choices with submerged platform at the end of all correct choices)



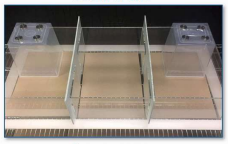
A: Source: Charles River Laboratories, Inc.
B: Original design by Ellen McGlinchey

Social interaction

- The 3-chamber test assesses cognition in the form of general sociability and interest in social novelty in rodent models of CNS disorders
- Rodents normally prefer to spend more time with another rodent (**sociability**) and will investigate a novel intruder more so than a familiar one (**social novelty**)
- Based on these inclinations, the 3 chamber test can help identify rodents with deficits in sociability and/or social novelty



Small arena



Large arena

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Terminal procedures

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Terminal procedures

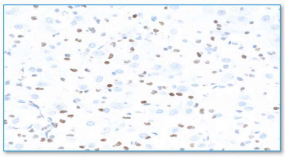
Morphometry (*e.g.*, lung)

Bone pathology, mechanics

Electron microscopy (*e.g.*, liver)

Neuropathology (CNS and PNS)

- Whole-body perfusion
- CNS
  - Paraffin embedding, special stains
- CNS/PNS
  - Plastic embedding, semi-thin sectioning, toluidine blue staining



Caspase 3 immunohistochemical staining (brown cells) reveals extensive apoptosis in the motor cortex of a rat at PND 6. This is part of the normal 'pruning' of the excess neurons that are present at birth in the rat.

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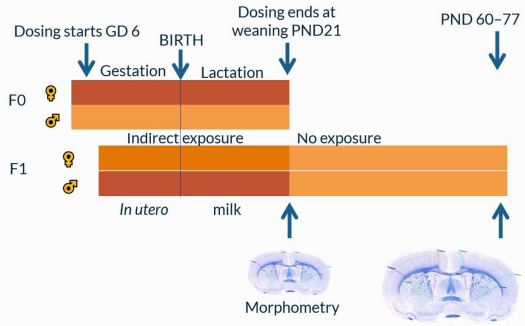
Developmental neurotoxicology



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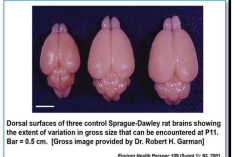
Brain landmarks

Routine H&E staining	For evaluation of all tissues
Bielschowsky's silver staining	For neurodegeneration evaluation: brain, spinal cord, gasserian ganglia, dorsal root ganglia
Toluidine-blue	For myelin integrity: peripheral nerve cross-sections
Luxol Fast Blue/Cresyl Violet (LFB/CV)	For myelin integrity and neuronal cytoarchitecture: Brain, spinal cord, gasserian ganglia, dorsal root ganglia

Tissue Examined	
Brain, including olfactory bulbs (all sections in their entirety)	Dorsal root ganglia and associated dorsal and ventral spinal nerve roots from the cervical, thoracic and lumbar regions of the spinal cord
Eyes	Peripheral nerves (sciatic, tibial, peroneal [fibular], and sural)
Gasserian ganglia (5th cranial nerves and ganglia)	Skeletal muscle (gastrocnemius and soleus)
Cervical, thoracic and lumbar regions of the spinal cord	

Source: Charles River Laboratories, Inc.



Dorsal surfaces of three control Sprague-Dawley rat brains showing the extent of variation in gross size that can be encountered at P11. Bar = 0.5 cm. [Gross image provided by Dr. Robert H. Garman]

Bolon, B. *et al.*, *Toxicol Pathol.* 2013;**41**(7):1028-1048

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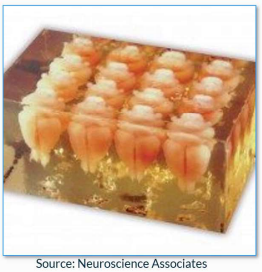
Brain landmarks



Source: Neuroscience Associates

Source: Charles River Laboratories, Inc.

Bolon, B. *et al.*, *Toxicol Pathol.* 2013;**41**(7):1028-1048

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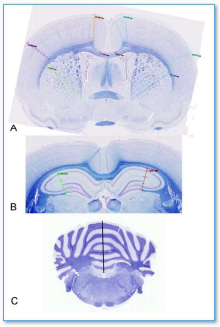
Brain landmarks

Levels (from Figure 1)							Brain structures (listed from rostral to caudal)
1	2	3	4	5	6	7	
X							Olfactory bulb
	X						Anterior commissure
	X						Septal nuclei
	X						Caudate/putamen
	X	X	X				Cerebral cortex (frontal, parietal, temporal, occipital)
	X	X					Corpus callosum
	X	X					Internal capsule
	X	X					External capsule
		X					Optic tract
		X					Amygdala
		X	X				Hippocampus
		X					Thalamus
		X					Hypothalamus
		X	X				Cerebral peduncles
			X				Midbrain, rostral
				X			Midbrain, caudal
				X			Pons
				X	X	X	Pyramids
				X	X	X	Cerebellum
					X		Deep cerebellar nuclei
			X	X	X	X	Reticular formation
					X	X	Trigeminal nuclei and tracts
						X	Medulla oblongata
		X				X	Choroid plexus



Bolon, B. *et al.*, *Toxicol Pathol.* 2013;**41**(7):1028-1048

Source: Charles River Laboratories, Inc.

Developmental neurotoxicology

- There are a lot of techniques that have been developed for DIT
- The functionality of the immune system can be tested
- Mouse is more thoroughly characterized however, DIT is most efficiently performed in rats
- DIT studies may be conjoined with existing DART protocols
- Histopathologic evaluation will have a role in detection of xenobiotic-associated immunomodulation although alone it will probably be insufficient

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