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PRIMATE BIOLOGICS RESEARCH AT A CROSSROADS

WAXMANN

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Contents

Preface

The 20th Covance Primate Symposium was held in a scenic and stimulating country-side location near Münster, Germany on May 20-21, 2014. It was a unique networking event that brought together leading scientists, experts and thought leaders in primate research, where approximately 100 participants convened from around the world. The theme – ***Primate Biologics Research at a Crossroads*** – put the focus on biologics, the fastest-growing area in drug development today. The nonhuman primate plays a pivotal role in a biologic's early development. However, we are at a crossroads of past and future in biologics, creating both a challenge and an opportunity to advance primate biologics research.

During the week, we explored best ways to accomplish success – and had an outstanding group of speakers that covered four key biologics topics:
• Immunogenicity Assessment: Challenges and Opportunities
• Evaluation of Neurotoxicity and CNS Functions
• Juvenile Toxicity Assessment Update
• Nonhuman Primate Disease and Efficacy Models

In addition, there were special opportunities for roundtable questions, break discussions and further debate during networking events to foster interactions and scientific discussions. Due to a number of unforeseen circumstances (altered job affiliation, intellectual property/privacy requirements) the book unfortunately does not contain chapters related to the first session topic on immunogenicity: however, a summary of this session is provided in the concluding remarks chapter.

The use of nonhuman primates in research continues to be under considerable public and legal scrutiny because the nonhuman primate is considered an evolutionary "higher" species compared to species such as rodents, rabbits, canines and minipigs. In Europe, there is a political effort to ban the use of nonhuman primates in (biomedical) research. However, from a scientific and human safety perspective, species selection must be driven by its relevance to predict human safety. While the debate continues, the presentations at this symposium have once again made it unequivocally clear that - at present - nonhuman primate models cannot be abandoned for biologics drug development. This is largely due to the fact that biologic drugs are substantially or fully humanized and frequently nonhuman primates provide the only relevant model. In addition, it is

imperative to stress that the applicable drug development guidance(s) require the use of *relevant* animal models.

The good news is that much progress has been made in reducing the number of nonhuman primates needed per biologic drug under development and, simultaneously, the experimental designs are evolving and being refined continuously. Some wonderful examples of 3R initiatives and achievements are shared in this book.

We would like to thank all of our presenters and chapter authors for their contributions. In addition, Andrea Greiter-Wilke for providing the very thorough and insightful concluding remarks for the symposium book proceedings. We are particularly indebted to Heidi Arendt for leading the local meeting organization team, and to the Covance marketing team for their continued and dedicated efforts which helped to deliver this successful event.

For the first time, the Covance Primate Symposium proceedings are being published as an eBook, permitting prolific digital access to the content, as well as, the use of coloured figures and micrographs. We would like to express our thanks to Dr. Ursula Heckel, representing the publisher, for her sustained flexibility and patience with chapter submission deadlines during the making of the 9th book of the Covance Primate Symposium series.

And, finally, to our readers, we now invite you to enjoy the science,

Gerhard F. Weinbauer
Friedhelm Vogel

From Science to Patients: The Role of Nonhuman Primates in Drug Development

Friedhelm Vogel

Abstract

Animal research – including research in the nonhuman primate (NHP) – is an essential part of the drug development process and has played an important role for the majority of medical achievements. As the EU is now stimulating the translation of basic research into therapies, this transition will often require the testing of experimental therapies in NHPs. On the other hand the EU wishes to restrict NHP experiments and European researchers start taking their NHP research outside of Europe, sparking a controversy that is dividing the scientific community, as the standards of ethical oversight and animal welfare could be lower in those countries compared to those in Europe.

The experimental use of NHPs in Europe is tightly regulated and is only permitted when there are no alternatives available. As a result, NHPs are most often used in late non-clinical phases of biomedical research. Although the impetus for scientists, politicians and the general public to replace, reduce and refine the use of NHP in biomedical research is strong, the development of 3R technology for NHP research poses specific challenges. On the other hand, there is a continued urgent need for new therapies which require the use of NHPs during the drug development process.

1. Introduction

Animal research is an essential part of the drug development process and has played an important role for the majority of medical achievements. Over the past twenty years, the development of monoclonal antibody therapies in particular has completely transformed our ability to treat diseases including breast cancer and other cancers, rheumatoid arthritis and multiple sclerosis, and the development of this technology would not have been possible without the use of animals – including nonhuman primates (NHPs) – both in developing the

fundamental elements of the technology and in producing the medicines used to treat patients.

Although NHPs make up less than 0.1% of the animals used in research, they play a key role in drug development due to their similarity to humans with regard to sensory organs, hormonal systems, reproduction, immune system etc., to evaluate efficacy and safety, especially for biopharmaceutical compounds. This leads to particular ethical considerations due to their phylogenetic closeness which is also reflected in the extensive application of the 3R concept – Replacement, Reduction, and Refinement.

Looking back, research in animals has always played a major role for achieving medical advances, and it is still a key component of the medicines development path, although it represents only approx. 5-10% of this process. Seven of the last 10 Nobel Prizes in medicine have relied at least in part on animal research, and Polio would still claim hundreds of lives every year without the animal research carried out by the Nobel laureate Albert Sabin. Although animals are still needed to test every new batch of polio vaccine produced for today's children, the original test requiring NHPs for neurovirulence testing of every single batch could be replaced by a test in a transgenic mouse model.

For only 10,000 out of 30,000 known diseases is there a treatment in place. Continuous research is required for further medical progress, and today this often involves biopharmaceuticals which pose a significant driver for NHP research. Important regulatory changes have been implemented to reflect these developments, particularly the following regulatory and guidance documents:

• European Union Directive 2010-63-EU on the protection of animals used for scientific purposes (translated into national laws)

• ICH Harmonized Tripartite Guideline M3 (R2):
  "GUIDANCE ON NONCLINICAL SAFETY STUDIES FOR THE CONDUCT OF HUMAN CLINICAL TRIALS AND MARKETING AUTHORIZATION FOR PHARMACEUTICALS"

• ICH Harmonized Tripartite Guideline S6 (R1):
  "PRECLINICAL SAFETY EVALUATION OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS"

But in parallel, the general political climate for animal research and particularly for NHP research has become very challenging and poses a considerable thread to NHP research in Europe. Animal rights groups apply increasing pressure on

research organizations and related industries with the goal to end all research in animals, not only in NHPs, and key targets are transport companies like airlines which represent a chokepoint in the NHP supply chain. These groups follow their ideological goals and take into account that animal research is forced into countries where the standards of ethical oversight and animal welfare may be considerably lower compared to those in Europe.

On the other hand, significant scientific advancements open up new opportunities for research and medical advancements, e.g. genetic profiling and personalized medicine, embryonic stem cells/iPS cells, and transgenic animal models including NHPs, created with cut-and-paste DNA (Niu et al. 2014; Sample 2014; Figure 1). Genome typing of NHP models is expected to have significant implications in the future (Haus et al. 2014).

The Guardian – 30 January 2014

Genetically modified monkeys created with cut-and-paste DNA

Breakthrough could help battle diseases such as Alzheimer's and Parkinson's but ethical concerns remain over animal testing

Ian Sample, science correspondent

theguardian.com, Thursday 30 January 2014 17.26 GMT

Figure 1:
Transgenic NHP Model (Sample 2014) © Guardian News & Media Ltd

2. Medical advances involving NHP research

In his foreword for the publication "Medical Advances and Animal Research" (RDS: Understanding Animal Research in Medicine and Coalition for Medical Progress 2007), Robert Winston states: "… Animal research is not done in isolation; it is one vital strand of medical research. … For honest, open debate we need to understand the role of research using animals in medical progress. … Tracing the research process – from 'blue-sky' or chance observation, through careful studies which include the use of animals for the ultimate benefit of people who are ill – will increase understanding of the essential endeavor."

John Illman states in the Introduction of the same publication: "Of course, animal models have limitations. They do not always adequately mimic human disease or responses to medicines. But they remain crucial and have made a major contribution to many of the biggest medical advances of our age."

Some prominent examples, involving research in monkeys, are listed in Table 1.

1930s	Modern anaesthetics Diphteria vaccine
1940s	Discovery of the rhesus factor Kidney dialysis
1950s	Polio vaccine Replacement heart valves
1960s	German measles vaccine
1980s	Life support systems for premature babies Hepatitis B vaccines Treatment for leprosy
1990s	Combined therapy for HIV infection New medicines for asthma
2000s	Deep Brain Stimulation to suppress symptoms of Parkinson's Disease
2010s	Hepatitis C vaccines

Table 1

Some further significant achievements were anti-rejection drugs for organ transplant recipients, surgical treatment for macular degeneration, new techniques in stroke habilitation therapy, drugs to combat asthma, and the first monoclonal antibody Herceptin (Genentech, Inc.) – just to name a few.

The development of Monoclonal Antibodies (mAbs) is an important part of today's research in drug development. While Herceptin (a humanized mouse protein) was the first one, developed for breast cancer treatment, other breakthrough mABs are Rituximab for treatment of lymphoma as well as rheumatoid arthritis, and Alemtuzumab for treatment of leukaemia.

In its report "Working to reduce the use of animals in scientific research" (UK Government 2014), the UK Government states: "The development of monoclonal antibody therapies over the last 20 years has completely transformed our ability to treat diseases including breast and other cancers, rheumatoid arthritis and multiple sclerosis. The development of this technology would not have been possible without the use of animals both in developing the fundamental elements of the technology and in producing the medicines used to treat patients."

Animals continue to play a critical role for medical advancements.

3. Animal statistics

According to the EU Report "COM(2013) 859 final" (European Commission 2013), 11.5 million animals were used for research in the European Union in 2011 (Figure 2), whereby three fourths were rodents. Only 0.05% were prosimians and monkeys; great apes were not used at all. Out of the 11.5 million animals, 8.75% were used for toxicological and other safety evaluation, i.e. for regulatory purposes within the drug development process (Figure 3).

Based on the UK statistics for animal experiments (UK Government 2014), the use of animals in research steadily increased since 1945 until the mid 1970's and then decreased until around 2000 (Figure 4). Since 2000, the numbers are increasing again. However, this has to be looked at in a differential way. As shown in Figure 5, the apparent increase is due to a change in methodology, as up to 1994 only non-breeding experiments in animals were counted. Since 1995, the breeding of genetically altered animals (mainly mice and fish) is also counting as scientific procedures and included in the statistics. However, it is obvious that the number of non-breeding experiments had further declined and is now ranging around 2 million per year (Figure 5).

A particular focus is on the use of animals which are very close to man, either because the species reflect companion animals like dogs and cats or because their obvious similarity to humans (i.e. primates). Again based on the UK statistics, the use of dogs and cats has significantly declined over the past years, while the use of primates had been pretty steady except for a steep decline in 2011/2012 which probably reflects temporary budget shifts due to the 2008/2009 financial crisis rather than a sustainable reduction (Figure 6), as the use of NHPs has since moved back to the pre-2011 run rate.
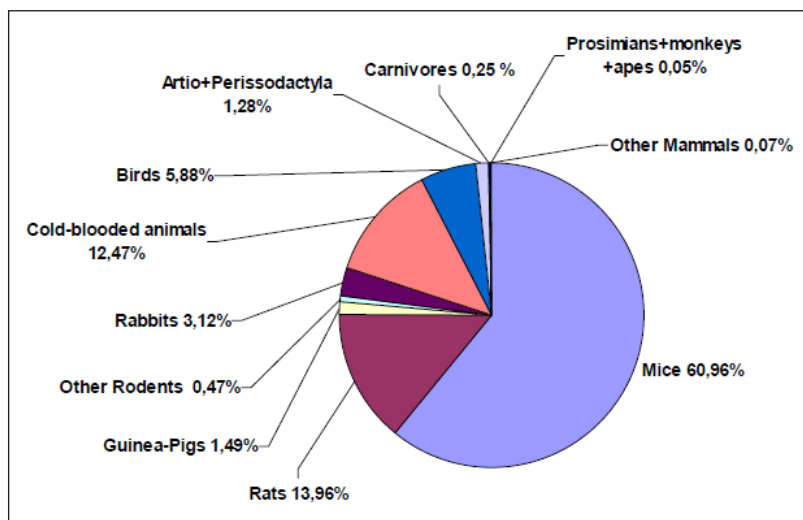
Figure 2:
Total no. of animals used in research in the EU in 2011 (European Commission 2013)
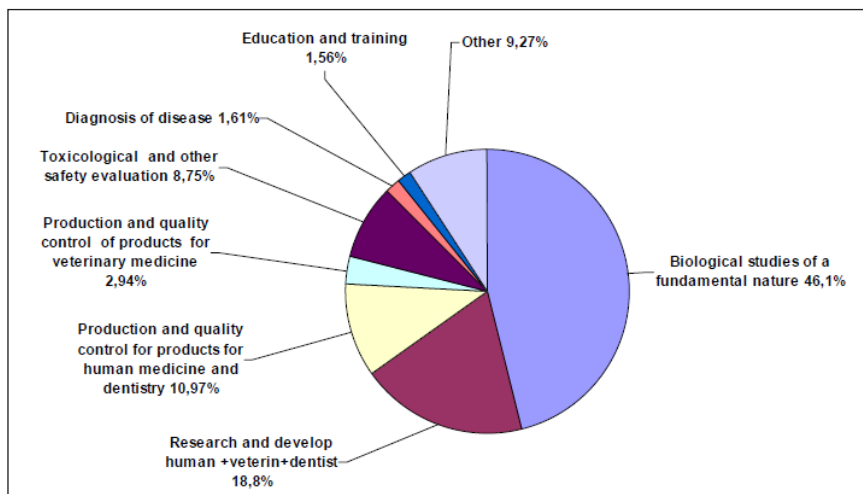
Figure 3:
Animals used in research in the EU in 2011 by purposes of experiments (European Commission 2013)
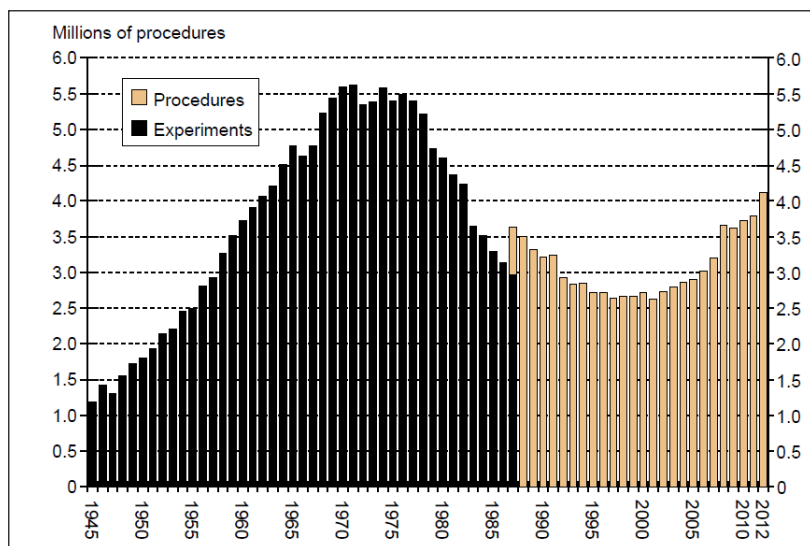
Figure 4:
UK statistic on animal experiments for 1945 to 2012 (UK Government 2014)
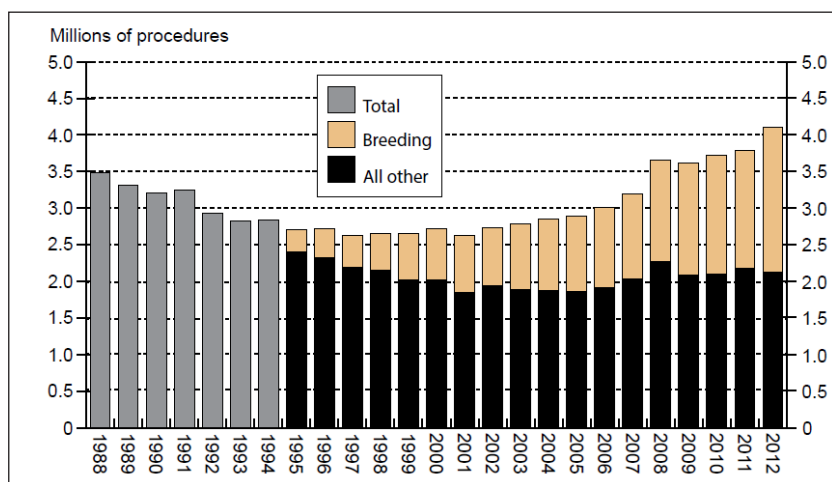
Figure 5:
UK statistic on animal experiments and procedures in 1988 to 2012 (UK Government 2014)
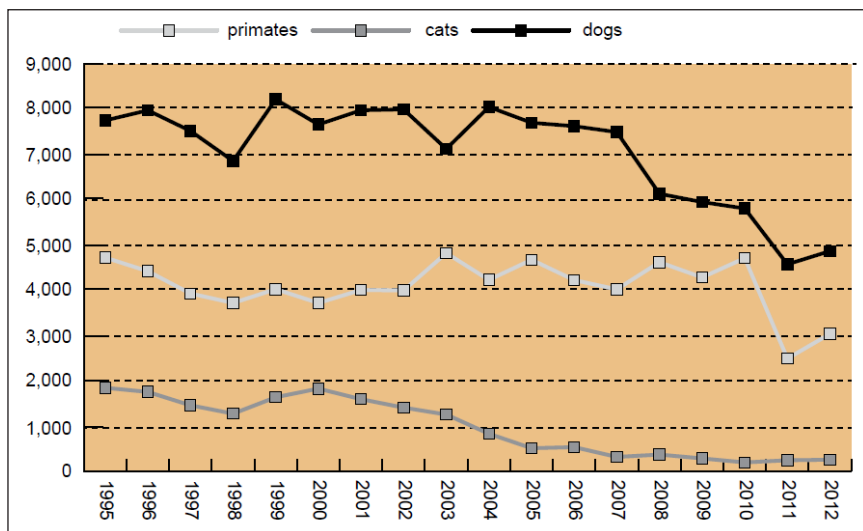
Figure 6:
UK statistic on animal experiments in dogs, cats and primates in 1995 to 2012 (UK Government 2014)

4. Predictability and efficiency of the drug development process

In spite of steadily increasing R&D expenditures for development of new drugs, the overall output, measured in approved NMEs (new molecular entities) and BLAs (biologics license applications) by CDER (US FDA Center for Drug Evaluation and Research) has remained fairly constant over the past years (Mullard 2014; Figure 7). Orphan drugs and cancer drugs dominated and only two novel monoclonal antibodies (mAbs) were approved in 2013 (Mullard 2014; Figure 7). 30% of FDA drug approvals were related to oncology (Mullard 2014; Figure 8).

At the same time, the efficiency and predictability of animal testing is challenged. In its report "Working to reduce the use of animals in scientific research", the UK Government (2014) states: "The scientific imperative for developing new approaches to research and development is very strong. Although the use of animals forms a major part of much scientific and medical research, success seen in animal studies has not always translated in the clinic. Many potential drugs fail due to lack of efficacy in humans or concerns about their safety. Methods are needed to screen these failures out as early as possible and
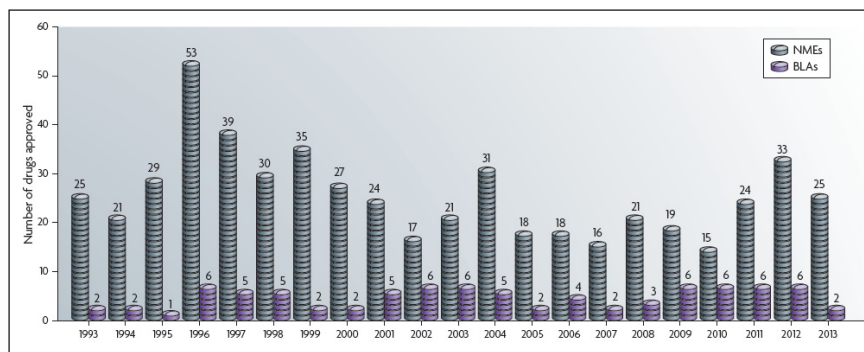
Figure 7:
FDA approvals since 1993 (Mullard 2014)
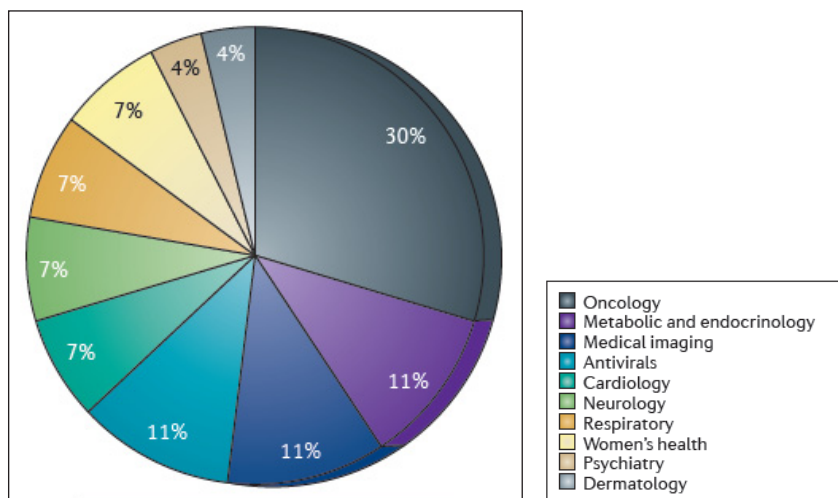
Figure 8:
2013 FDA approvals by therapeutic area (Mullard 2014)

to select, with further research and development, those approaches most likely to succeed."

Hay et al. (2014) analysed clinical development success rates across the industry and came to the conclusion that "achieving FDA approval for only one-in-ten drug indications that enter the clinic is a concerning statistic for drug developers, regulators, investors and patients." Figure 9 shows the success rates
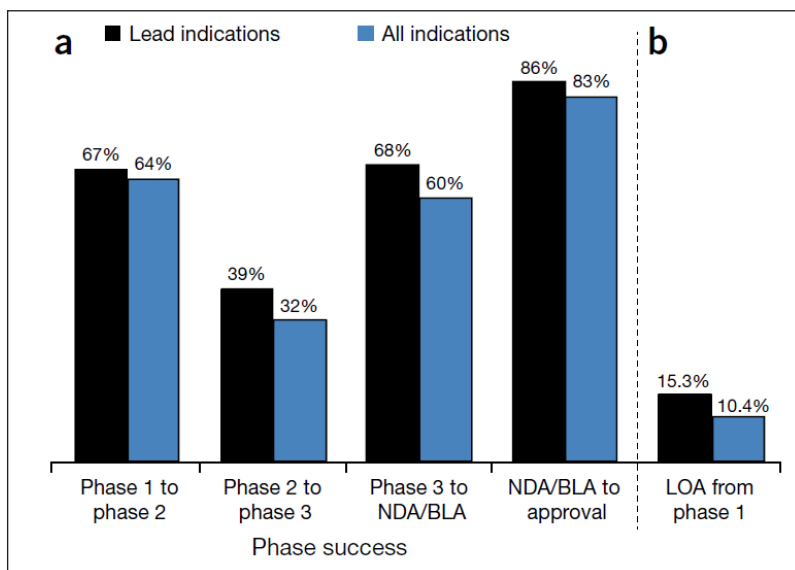
Figure 9:
Clinical phase success rates and likelihood of approval (Hay et al. 2014)

between the different phases of clinical development as well as the overall success rate throughout clinical development, whereby the success rates for lead indications were slightly higher than for all indications. Success rates varied by disease indications (Figure 10).

Based on the root cause analysis performed, Hay et al. (2014) determined that the main causes for discontinuation or suspension of development programs in Phase 3 of clinical development or upon NDA/BLA were insufficient efficacy or insufficient safety (Figure 11). They suggested that "for example, more predictive animal models, earlier toxicology evaluation, biomarker identification and new targeted delivery technologies may increase future success in the clinic" (Hay et al. 2014).

Olson et al. (2000) summarized the results of a multinational pharmaceutical company survey and the outcome of an International Life Sciences Institute (ILSI) Workshop in April 1999, to better understand concordance of the toxicity of pharmaceuticals observed in humans with that observed in experimental animals. The survey included input from 12 pharmaceutical companies with data
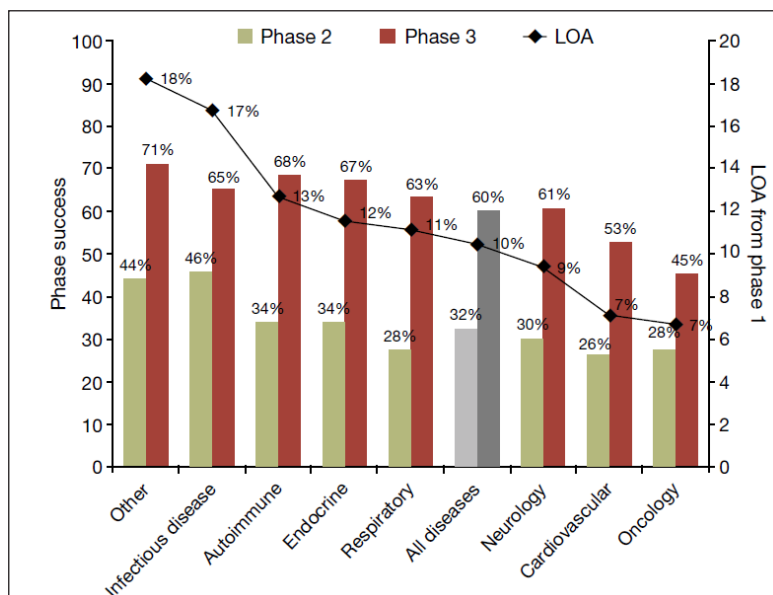
Figure 10:
Phase success and likelihood of approval from phase 1 by disease indications (Hay et al. 2014)

compiled from 150 compounds with 221 human toxicity events (HT events) reported; multiple HT events were reported in 47 cases. The main aim of the project was to examine the strengths and weaknesses of animal studies to predict human toxicity (HT).

"The results showed the true positive HT concordance rate of 71% for rodent and nonrodent species, with nonrodents alone being predictive for 63% of HTs and rodents alone for 43%. The highest incidence of overall concordance was seen in hematological, gastrointestinal, and cardiovascular HTs, and the least was seen in cutaneous HT. Where animal models, in one or more species, identified concordant HT, 94% were first observed in studies of 1 month or less in duration. These survey results support the value of in vivo toxicology studies to predict for many significant HTs associated with pharmaceuticals and have helped to identify HT categories that may benefit from improved methods" (Olson et al. 2000).
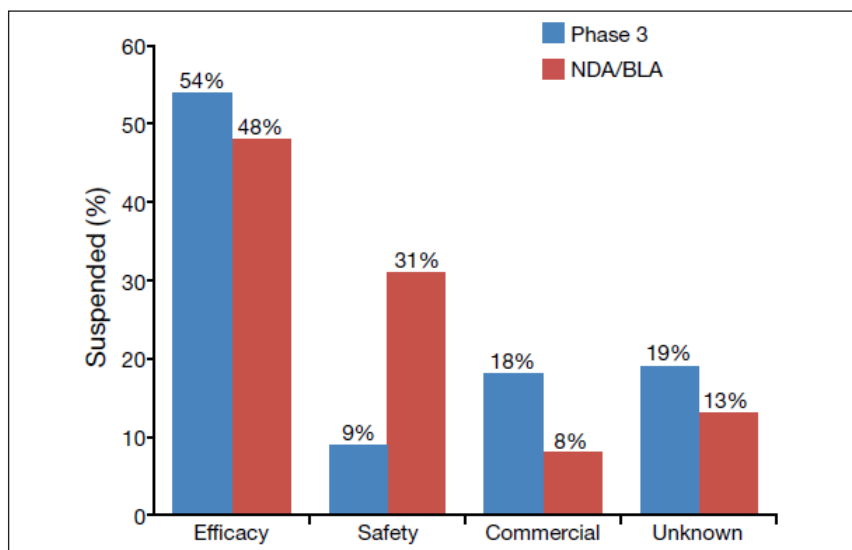
Figure 11:
Root cause analysis for suspension of development programs (Hay et al. 2014)

5.   Research in nonhuman primates

The NHP continues to be a very valuable animal model in drug development, particularly for biopharmaceuticals, where the phylogenetic closeness and particular similarities in metabolism, sensory organs, hormonal system, reproduction, immune system etc. as well as highly developed social skills are of specific importance. Species specificity can be crucial for some compound classes, e.g. mABs. For some disease indications, specific NHP models were developed which successfully mimic the disease in humans, e.g. for Graves' disease (Wang et al. 2013).

Another advantage of the NHP as model in toxicology is that methodologies from the clinical environment can often directly be transferred to and applied in NHPs, e.g. intrathecal dosing techniques, electroretinography in compliance with international clinical standards etc.

According to the MRC & Wellcome Trust Report "Primates in Medical Research" (2006), there are six key areas of NHP research:

- Parkinson's disease:
  - due to similarity of brain pathways for the control of movement between monkeys and humans;

- Stroke:
  - due to structural brain similarities;

- Reproduction:
  - as NHPs are the only mammals that ovulate and menstruate every month;
  - as NHPs may show early miscarriage;
  - as endometriosis is observed in NHPs;
  - due to occurrence of the polysystic ovary syndrome;
  - as problems with menstrual bleeding can occur;

- Cognition:
  - as we can focus on basic processes underlying how we think, understand and remember;
  - as primates are the only animals which can learn, remember and perform complex tasks in a way that mirrors human activity;

- Vaccines:
  - due to the similarity in immune response processes, e.g. in HIV research or Hepatitis C research;

- Vision:
  - due to similarity in eye morphology and physiology between primates and human, also in regard to areas of the brain involved in vision.

The use of NHPs in biomedical research is justified and essential when the benefits are great and outweigh ethical considerations and when there are no realistic alternatives. This is also reflected in the Directive 2010/63/EU which explicitly states that the use of NHPs "should be permitted only for basic research, the preservation of the respective non-human primate species or when the work, including xenotransplantation, is carried out in relation to potentially life-threatening conditions in humans or in relation to cases having a substantial impact on a person's day-to-day functioning, i.e. debilitating conditions."

6. From research to medicines

There still exist many "pharmaceutical gaps" for various diseases, diagnostics, and conditions, as outlined in the 2013 Report "Priority Medicines for Europe and the World" (Kaplan et al. 2013; see Figure 12). This is an update to the original 2004 report and provides a public-health-based medicines development agenda, taking into account increasing life expectancy (Figure 13) and related population aging (Figure 14). Bioresearch, probably the most important area of NHP research, is considered essential for medical progress, and the report (Kaplan et al. 2013) outlines various key findings and recommendations, especially:
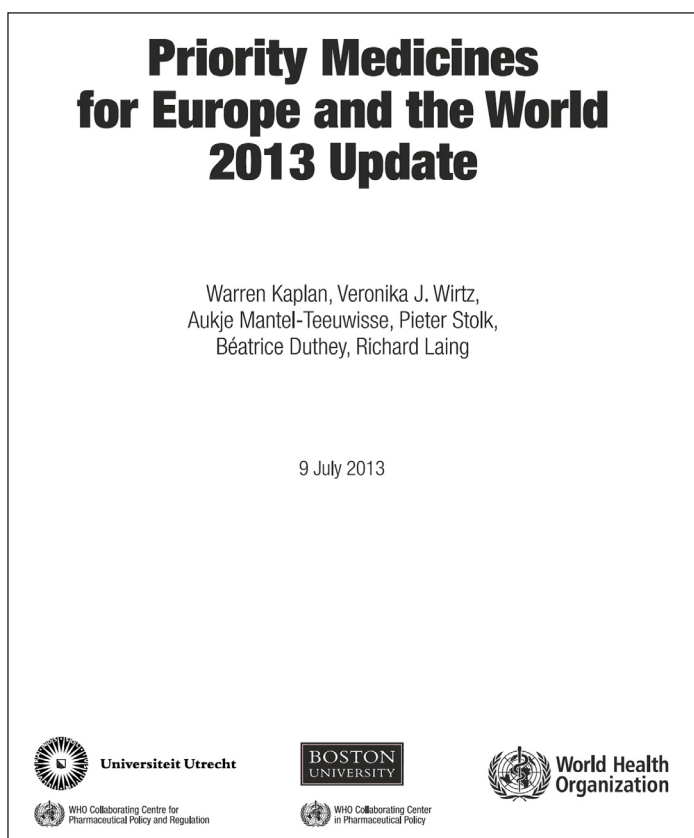
Figure 12:
2013 Report "Priority Medicines for Europe and the World" Kaplan et al. 2013
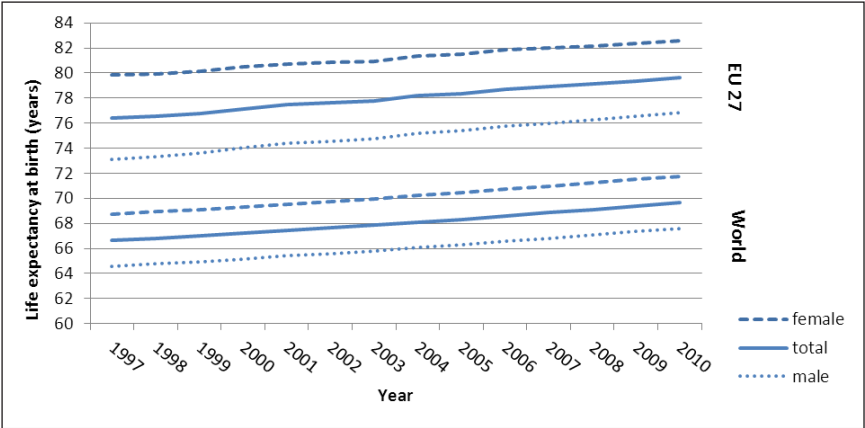
Figure 13:
Life Expectancy in Europe (EU 27) and in the World
Source: Data from *the World Bank. World Development Indicators.* Available at: http://databank.worldbank.org
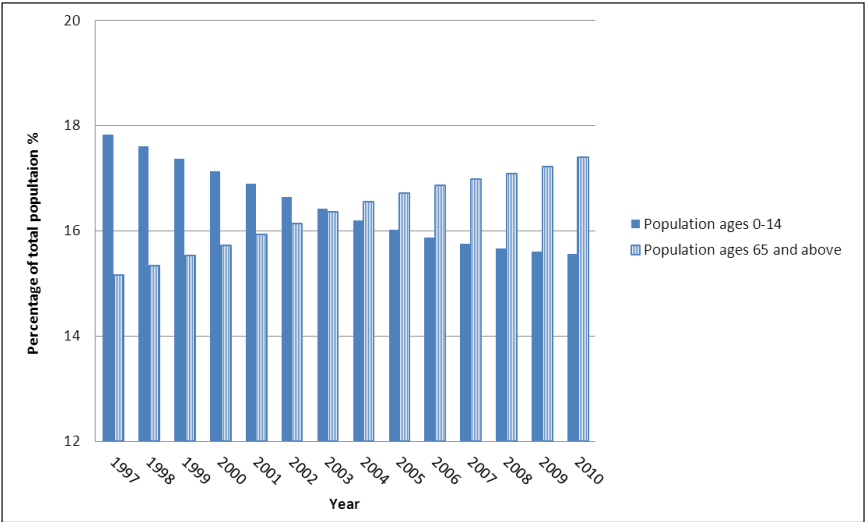
Figure 14:
Aging Population
Source: Data from *the World Bank. World Development Indicators.* Available at: http://databank.worldbank.org

- "The population of Europe and the world is ageing, with more people – especially women – living beyond the age of 80."
  - Marked increase in diseases of the elderly (e.g. Alzheimer, osteoarthritis, hearing loss).

- "Ischaemic heart disease and stroke are the laregst contributors to the European burden of disease and among the leading contributors to the global burden of disease."
  - Optimise secondary prevention (polypill).
  - Large clinical trials needed.

- "Stroke, osteoarthritis, Alzheimer disease, hearing loss, low back pain, chronic obstructive pulmonary disease (COPD) and alcoholic liver disease are seven high-burden conditions, in Europe particularly, for which the currently available treatment is inadequate in reversing or halting the progression of disease."
  - New medicines required and improvement of existing medicines.

- Identification of biomarkers for many diseases.
  - Identify potential products, diagnose and monitor disease progression, assess treatment effects.

- "Antibacterial resistance [***AMR***] and pandemic influenza remain major threats to global public health which require a coordinated international effort."
  - New diagnostic tests, new R&D models, and prevention through vaccination required.

- "Malaria and tuberculosis (TB) represent major threats, especially in low- and middle-income countries; TB is also an important disease in some European countries."
  - Same challenge as with AMR/pandemic flu: resistance will remain a threat until primary prevention (vaccination) occurs.

- "Diarrhea, pneumonia, neonatal conditions and maternal mortality are major contributors to the global burden of disease."
  - Improvement of diagnosis and treatment required, including reducing costs.

- "Stratified Medicine, in which specific patient groups are identified who would benefit most from particular therapies, will need to be carefully re-

searched over the next decade."
– From one-size-fits-all to targeted treatments and personalized medicine.

This medicines development agenda has a significant impact on pharmaceutical research and development and is also changing the face of primate research. The EU is pushing "for the translation of basic research into therapies – a transition that often requires the testing of experimental therapies in primates. And opportunities for translational research are growing thanks to recent technological breakthroughs. However, restrictions on primate experiments could hinder their development" (Abbott 2014). This will require appropriate and reliable animal models and in many cases NHP models.

On the other hand however, the EU wishes to restrict NHP experiments, and European researchers are now taking their NHP research outside of Europe, sparking a controversy that is dividing the scientific community. The standards of ethical oversight and animal welfare could be lower in those countries compared to those in Europe, especially with regard to the 3Rs – Replacement, Reduction, and Refinement – a key imperative for research in the Western world. State-of-the-art NHP research and 3Rs go hand in hand, and the scientific community has to speak up in public to ensure that essential and critical research is not pushed out to countries of lower standards – at the disadvantage of animals in research.

7. Conclusion

The experimental use of NHPs in Europe is tightly regulated and is only permitted when there are no alternatives available. As a result, NHP are most often used in late non-clinical phases of biomedical research. Although the impetus for scientists, politicians and the general public to replace, reduce and refine the use of NHPs in biomedical research is strong, the development of 3R technology for NHP research poses specific challenges. On the other hand, there is a continued urgent need for new therapies which require the use of NHPs during the drug development process.

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Neurobehavioural Assessment of Biopharmaceuticals in Nonhuman Primate

Alessandra Giarola

Abstract

Biotechnology-derived pharmaceuticals differ from small organic molecules in their method of synthesis, size and the high degree of specificity of target interactions and they require the application of unique approaches to assess their safety in preclinical studies. Because of these differences, the ICH S7A guidance document treats these molecules separately and states that it is often sufficient to evaluate safety pharmacology endpoints as a part of toxicology and/or pharmacodynamic studies; therefore, safety pharmacology studies can be reduced or eliminated for these products. The decision whether or not to perform a standalone functional observational battery or include it in the general toxicology studies needs to be based on a risk assessment that takes into consideration the characteristics of the biopharmaceutical compounds, the pharmacodynamic and pharmacokinetic characteristics, the indication and the study design for the general toxicology study. Each compound has unique characteristics that will influence the development strategy and therefore requires a science-based, case-by-case, approach to evaluation of its safety pharmacology.

1. Introduction

The introduction of biotechnology-derived pharmaceuticals for clinical use requires the application of unique approaches to assess their safety in preclinical studies (Baumann 2014). There is much diversity among these products, which include gene and cellular therapies, monoclonal antibodies, human-derived recombinant proteins, blood-derived products and vaccines. For many biological therapies there will be unique product issues that may require a differential development and may raise safety concerns (Serabian et al 1999). Non clinical safety testing of new biopharmaceutical entities represents its own challenges and opportunities in drug development. All new pharmaceutical and biophar-

maceuticals must undergo non clinical safety testing prior to human administration. Safety pharmacology (SP) assessment of small molecules is generally conducted in standalone studies that appropriately designed to detect small changes (Pugsley et al 2008). One important consideration for biotherapeutics is species selection and the assurance that the drug is pharmacologically active in the selected preclinical species. The issue of species specificity means that nonhuman primates (NHPs) play an important role in the development of biotechnology products and as they are often the only relevant species that can be used to assess the safety of biotherapeutics (Chapman et al 2007; Bussiere 2008). Other challenges are the novelty of the molecules that are currently under development, the unique delivery systems, use of alternative routes of administration, long half-life and mismatch between pharmacokinetic and (PK) and pharmacodynamic (PD).

2. Regulatory framework for biopharmaceuticals

The ICH S6(R1) guidance "Preclinical safety evaluation of biotechnology-derived pharmaceuticals " is the only regulatory guidance that specifically addresses nonclinical safety evaluation of biotechnology-derived products and provides a flexible, rational, science-based framework for testing their preclinical safety. It is not prescriptive and recognises the value of alternative approaches: "*It is important to investigate the potential for undesirable pharmacological activity in appropriate animal models and, where necessary, to incorporate particular monitoring for these activities in the toxicity studies and/ or clinical studies. Safety pharmacology studies measure functional indices of potential toxicity. These functional indices may be investigated in separate studies or incorporated in the design of toxicity studies. The aim of the safety pharmacology studies should be to reveal any functional effects on the major physiological systems (e.g., cardiovascular, respiratory, renal, and central nervous systems)" (ICH S6(R1) 2011).*

Preclinically the assessment of Central Nervous System (CNS) and Peripheral Nervous System (PNS) safety is particularly important for all new potential pharmaceutical compounds and it has been included as part of the "core battery' assessment of vital organ functions in the ICH S7A guideline for SP (ICH S7A 2001). In particular, it is mentioned that "Effects of the test substance on the central nervous system should be assessed appropriately. Motor activity, behavioural changes, co-ordination, sensory/motor reflex responses and body temperature should be evaluated. For example, a functional observational battery (FOB), modified Irwin, or other appropriate test can be used."

The ICH S7A guideline recognises some exceptions for not performing safety pharmacology studies (dermal or ocular locally applied agents, cytotoxic compounds) and states that for biotechnology-derived products that achieve highly specific receptor targeting, it is often sufficient to evaluate safety pharmacology endpoints as a part of toxicology and/or pharmacodynamic studies, and therefore SP studies can be reduced or eliminated. On the other hand the same guidance highlights that for biotechnology-derived products that represent a novel therapeutic class and/or those products that do not achieve highly specific receptor targeting, a more extensive evaluation by SP studies should be conducted.

3. Neurobehavioural assessment in nonhuman primate

The so called "Irwin test" was introduced in the pharmaceutical industry initially as a rapid psychotropic screening procedure adapted from those described first by Samuel Irwin for detecting behavioural effects in mice (Irwin 1968), it was subsequently modified to form Functional Observational Batteries (FOB) (Moser et al 1988; Mattsson et al 1996; Redfern et al 2005; Moscardo et al 2007) for use in other rodents (e.g., rats). Neurobehavioural batteries have been more recently adapted for use in the most commonly used non-rodent species in safety assessment studies, such as the dog (Gad et al 2003; Tontodonati et al 2007; Moscardo et al 2009) and the NHP (O'Keeffe et al 1989; Haggerty 1991; Gauvin et al 2008; Moscardo et al 2010).

NHPs possess more similarities to humans when compared to other/lower species in terms of the nervous system's anatomy, morphology and functional processes. Therefore NHPs are generally considered to be the most suitable experimental animals for research on higher-level cognitive and behavioural processes. The neurobehavioural assessment in NHPs can be used to evaluate the toxicity of pharmaceutical and other products in specific cases. The NHP FOB has been adapted and developed from the rodent and dog FOBs (Moscardo et al 2006). Different standardised and detailed observation grids adapted to the NHP (of defined strain and age) have been developed, that consists in a series of observations/measurements, including behavioural observations (in the home cage and examination room) and physiological/neurological measures (Table 1) (Gauvin et al 2008; Moscardo et al 2010). The NHP has high inter-individual behavioural variability, therefore normal behaviour needs to be established by detailed knowledge of each individual animal. The number of behavioural evaluations that can be standardised in the NHP is less compared to those in rodents, and therefore it is more important (compared to other species) to in-

Table 1:
Example of direct functional observational test battery in the monkey with scoring system. A/P* indicates sign is recorded as absent (A) or present (P). (Moscardo 2010)

	OBSERVATION	NORMAL SCORE	SCORERANGE
Assess with animal inside the homecage, observations only	Posture	4	0-8
	General locomotor activity	4	0-8
	Balance and co-ordination	4	0-4
	Alertness, general arousal state	4	0-8
	Vocalisations	4	0-8
	Groorning	4	0-8
	Tremors	0	0-4
	Convulsions	0	0-4
	Twitches and jerks	0	0-4
	Unusual behaviours/stereotypes	0	0-4
	Salivation	4	0-8
	Lachryrnation	4	0-8
	Ptosis	0	0-4
	Piloerection	0	0-4
	Mucosae	4	0-8
	Urination	A/P*	NA
	Defaecation	A/P*	NA
	Retching/vomiting	0	0-4
	Startle Response	4	0-8
	Aggressiveness	4	0-8
Observations with animal outside the cage using hand restraint	Ocular motility (voluntary and involuntary)	0	0-4
	Ocular position/symmetry	0	0-4
	Pupil diameter	4	0-8
	Pupilary reflex	4	0-8
	Blink reflex	4	0-8
	Limb (muscle) tone	4	0-8
	Flexor reflex	4	0-8
	Extensor reflex	4	0-8
	Respiration rate		
	Depth of respiration		Shallow, normal, deep
	Fine motor control and grip	4	0-4

tegrate neurobehavioural responses with clinical signs derived from a general toxicological assessment. In this way the progression/regression of the sign can be followed for a long period and the data used to decide the need to repeat the neurobehavioural battery on a case-by-case basis (Haggerty 1991). Some other important characteristics of the physiology and behaviour of the NHP need to be taken into account when the FOB is designed. NHPs normally live in structurally organised social groups, exhibiting a range of behaviours involving dynamic interactions with other group members, which may be directly comparable with those in humans. In addition, due to their complex central nervous system, NHPs show high cognitive functions and behavioural processes that are difficult to evaluate, or absent in other lower species. In particular, memory impairment involving the identification of another group member is a peculiarity and is directly analogous to humans. In NHPs the adult repertoire of vocalizations comprises a number of different calls, used to indicate territoriality, aggression, alarm, fear, contentment, hunger, the presence of food, or the need for companionship. It is, therefore important to evaluate the presence and normality of these characteristic vocalizations, as they have proved particularly valuable tools for examining cognitive processes (e.g., checking and communicative vocalizations) (Zuberbuhler 2006). Pharmacological responsiveness may be affected by social variables, group-housed animals provide more realistic models for assessing effects on human behaviour. For social species and in particular NHPs, extended solitary housing (e.g., study design that requires single housing) may result in behavioural abnormalities (e.g. abnormal gait, limb-jerking and head-jerking stereotypes and other bizarre or self-injurious behavioural patterns). These observations lead to the conclusion that NHPs should be group-housed from birth in a typical group structure (O'Keffee et al 1989).

4. Neurobehavioural assessment for biopharmaceuticals

In an industry survey published by Authier it was reported that for new chemical entities (NCE) the SP measurements in toxicology studies were conducted in addition to standalone SP studies (by 40.6% of the responders) or in addition or instead of standalone SP studies (by 39.8% of the responders). For biopharmaceutical agents, a majority indicated SP measurements in toxicology were conducted instead of standalone studies (74.3%) while inclusion of SP in toxicology studies for biopharmaceuticals in addition to standalone studies was reported in 25.7% (Authier et al 2013). The inclusion of SP endpoints into toxicology studies is generally accepted for biologic agents as they represent compounds with low risk profile (high affinity for the efficacy target and low incidence of off-target binding). Such considerations change for biopharma-

ceutical that cross the blood brain barrier (BBB). The ability to evaluate the SP endpoints after repeat dosing that results in cumulative effects is advantageous. Although pharmacologically driven adverse effects of a substance may be detectable at exposures that fall within the therapeutic range in appropriately designed SP studies, they may not be evident from observations and measurements used to detect toxicity in conventional toxicity studies, especially if the general toxicology effects mask the pharmacological subtle effects (Luft et al 2002). Logistical issues due to numerous activities ongoing in the examination room during the conduct of general toxicology studies needs to be taken into consideration as this may lead to a loss of sensitivity of the SP endpoints. Selection of proper SP endpoints for use in these studies is deemed important to minimise the interference from toxicology studies. In particular in the general toxicology studies in NHPs the number of simultaneous activities going on in a single day is high (e.g. blood sampling, ECG, blood pressure measurement etc) and the risk of adversely influencing the outcome of the CNS evaluation is significant. The risk of jeopardising the CNS assessment can be mitigated by considering the following key points:

• Neurobehavioural assessment performed in a quiet room with minimum personnel;
• FOB performed always prior to any procedures that can cause stress or discomfort (e.g. blood sampling, blood pressure measurements, ophthalmoscopy etc);
• Well trained technicians;
• NHP should undergo a training to the procedures and the personnel;
• Good communication among the team.

The decision whether or not to perform a standalone FOB or include it in the general toxicology studies needs to be based on a risk assessment that takes into consideration the characteristics of the biopharmaceutical compounds (e.g. CNS penetrant or not), the PK and PD characteristics, the indication, the study design for the general toxicology study and the route of administration (conventional vs non-conventional). Because each compound has unique characteristics that will influence the development strategy, the SP evaluation requires a science-based, case-by-case approach (Vargas et al 2013). The limitations of conducting pharmacological measurements in regulatory toxicology studies are acknowledged and the integration of SP endpoints into general toxicology studies is laborious. This disadvantage is counterbalanced by an expanded data set, larger number of experimental animals available, integration between SP, toxicological, PK, PD parameters. In addition the opportunity to test the compounds after chronic treatment and during recovery is an added value as the

Table 2:
Example of 3 weeks intravenous administration dose toxicity study followed by recovery period with integrated functional observational battery. [1] Blood sampling for toxicokinetic (TK); [2] Electrocardiography exam (ECG); at day and 1 and 14 ECG is measures continuously for 24 hours; [3] Blood Pressure measurement (BP); at day 1 and 14 is measures twice a day; [4] Functional Observational Battery (FOB); # extra timepoints can be selected in a case-by-case basis.

Day	Dosing	Clinical examination	Body-Weight	TK[1]	Ophthal-mology	ECG[2]	BP[3]	FOB[4]
Pre-treatment		X	X	X	X	X	X(2tlmes)	X (possibly arrange a dedlcated day)
Pre-dose		X	X		X	X	X(2times)	
Day of Treatment 1	X	X	X	X			X(24h)	X(2times)	
2	X	X							X (before the clinical examination and body weight)
3	X	X							
4	X	X							
5	X	X							
6	X	X							
7	X	X							
8	X	X							
9	X	X							
10	X	X							
11	X	X							
12	X	X							
13	X	X							
14	X	X	X	X	X	X(24h)	X(2tlmes)	
15	X	X							X (before the clinical examination and body weight)
16	X	X							
17	X	X							
IS	X	X							
13	X	X							
20	X	X							
21	X	X							
Start of recovery 1		X	X	X					
2		X							X (before the clinical examination and body weight)
3		X							#
4		X							#
5		X							#
6		X							#
7		X	X	X					

pharmacodymanic effect of biopharmaceuticals is often long lasting and therefore included as part of the study.

A typical SP study design for a biopharmaceuticals is a parallel design taking into account the long half-life, includes three tested doses and vehicle. Timepoints for the direct neurobehavioural exam are generally performed pre-dose (at approximately the same time of the day as the expected Tmax) at the estimated Tmax and at 24 hours post dose (to evaluate delayed effects and recovery, based on PK profile). Any additional timepoints can be added based on the PK and PD profile (Table 2).

5. Conclusions

The performance of standalone FOB in NHP can ensure the best level of performance of the neurobehavioural assessment as the different factors that can potentially jeopardise the assessment are under control (e.g. no other concomitant stressful activities, trained personnel, trained animals etc). If the study design is tailored to the compound characteristics and appropriately designed, the inclusion of the FOB in the general toxicology studies is a good compromise and has some advantages. An alternative option can be performing FOB in non-GLP study using that as an early assessment to trigger any further follow-up studies to drive the decision whether or not to perform a standalone SP study or SP endpoints integrated in general toxicology studies. There are also other challenges that need to be taken into account such as including CNS SP endpoints for a compound with prolonged Tmax and/or which accumulates upon repeated administration, or when active metabolites need to be considered as well. The situation is easier for compounds with rapid clearance, as every day of treatment can replicate the same conditions as Day 1.

In conclusion, biopharmaceuticals are unique therapeutic agents which require a science-based flexible approach in regard to the execution of SP studies. FOB results cannot be treated in isolation but need to be integrated with other data available (e.g PK/PD characteristics, in vitro pharmacology binding, in vivo data coming from other studies). It is important to mention that the reliable integration of SP endpoints in the general toxicity study allows a reduction in the number of animals used, which represents another important advantage from an ethical perspective when NHPs are used in preclinical development of biopharmaceutical products.

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Nonhuman Primate Models of Abuse Liability

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Abstract

This chapter highlights the use of nonhuman primates (NHPs) in models of abuse liability. First, we describe the two most frequently used models of abuse liability: drug discrimination and drug self-administration. We then provide several examples, all in NHPs, of how these models can address issues related to the regulatory (in this case the United States Food and Drug Administration and Drug Enforcement Agency) scheduling of novel compounds. The use of NHPs allows for longitudinal, within-subject designs in which multiple drugs can be evaluated, under identical conditions, to provide a better understanding of the pharmacological variables influencing abuse liability; such information cannot be obtained in rodent models. Finally, we discuss the advantages of using NHPs in assessing treatment compounds for cocaine abuse.

1. Introduction

Novel drugs are scheduled based on several factors, the first of which is a compound's "actual or relative potential for abuse" (FDA 2010). If a drug has some medicinal purpose but high abuse potential, it is designated Schedule II. The lower the abuse potential, the higher the number. For example, Schedule V drugs have much lower abuse liability than Schedule II drugs. Abuse potential can be determined in multiple ways, and we will focus on the two methods most frequently employed: drug discrimination and drug self-administration. Drug discrimination models "subjective-like effects" of drugs, while self-administration assesses the ability of drugs to maintain behavior leading to their presentation. Animals, primarily rats and monkeys, self-administer most of the drugs humans abuse and, in many cases, the use of rodent and monkey models provides similar information. There are cases, however, when species differences emerge and the focus of this review will be on describing scenarios when it is advantageous to use NHPs for these assessments. We will describe situations in which data obtained in NHPs provide novel information that would be difficult to obtain from other animal models. The importance of this distinction is

that the FDA does not require NHP models for abuse liability assessments and we will argue that there are cases when NHPs should be the species of choice.

All drugs, including drugs that do not have known abuse potential, can have three stimulus effects: unconditioned, discriminative and reinforcing. Unconditioned stimulus (US) effects are those that require no training to observe a response (termed an unconditioned response, UR). The UR could be (1) physiological, such as heart rate, blood pressure, body temperature, (2) neurochemical, which could be measured with in vivo microdialysis, or (3) behavioral, such as stereotypy, blinking, yawning or scratching. In pharmacology, these URs do not always appear in rodent species, thereby omitting a potentially critical piece of information related to the compound under investigation. For example, high doses of nicotinic agonists can induce emesis (Benowitz 1988; Tonstad et al 2014), but rodents do not have this UR and therefore, the study of nicotinic compounds in rats or mice will be devoid of data related to this potential side effect. A related point regarding UR is that monkeys can be implanted with indwelling telemetry devices and studied for *years* allowing for the comparison of drugs on physiological measures, as well as the determination of consequences of long-term drug use on these measures, perhaps indicating tolerance or sensitization to chronic drug exposure. The advantages of using NHPs to study the discriminative stimulus effects of drugs are based on the observation that drug discrimination (DD) models central nervous system (CNS) effects and the NHP CNS is more similar to humans than the rodent CNS. Animal models of self-administration are the most predictive models of human disease – animals will self-administer many of the same drugs that humans abuse and by the same routes. NHPs, but not rodents, can be trained to smoke nicotine, cocaine, heroin and hashish (Pickens et al 1973; Ando and Yanagita 1981; Carroll et al 1990; Evans et al 2003). An important variable that would impact all three of these stimulus properties of drugs that should be taken into consideration for human abuse liability assessments is the pharmacokinetic differences between rodents and NHPs. For instance, the onset and duration of action can significantly impact the reinforcing effects of a drug and consequently influence the rate of drug self-administration (Winger et al 1975; Panlilio and Schindler 2000). Given the more similar physioloigcal processess related to drug pharmacokinetics between NHPs and humans, NHP models of drug abuse may hold greater predictive validity as it relates to abuse liability assessments. As we describe below, another advantage of NHPs is the ability to study the same subjects across a range of drugs, thereby providing information relating phenotype to pharmacological outcomes.

Most of the studies described in this review will involve Old World macaques: rhesus and cynomolgus monkeys. As described elsewhere (e.g., Weerts et al 2007; Czoty et al 2015), there are several advantages to using NHPs in research. There are differences in brain anatomy between rodents and primates (including NHPs), making NHPs ideal for translational longitudinal neuroimaging studies (e.g., Morgan et al 2002; Nader et al 2006; Czoty et al 2007; see reviews by Howell and Murnane 2011; Murnane and Howell 2011; Nader and Banks 2014). In addition to neuroanatomy, there are neurochemical differences, including the dopamine (DA) neurotransmitter system, which is considered the primary neurotransmitter mediating drug reinforcement (Kalivas and Volkow 2005; Koob and Volkow 2010). For example, there are documented differences in brain DA systems and the response to drugs of abuse between rodent and NHPs (Berger et al 1991; Levant 1998; Joel and Weiner 2000; Porrino et al 2002). Other advantages include the study of prenatal drug exposure, in which Old World macaques have an approximate 6-month gestation period (Silk et al 1993), developmental differences (e.g., adolescent drug use; see Gill et al 2012; Soto et al 2012), social factors (e.g., Morgan et al 2002; Nader et al 2012) and the study of sex differences since female macaques have an approximate 28-day menstrual cycle with similar hormone fluctuations as women (Appt 2004).

2. Drug discrimination models

In models of drug discrimination, the subject uses the presence or absence of the training drug as a cue for making correct responses. For example, if the investigator administers a dose of 0.2 mg/kg cocaine (the training drug and dose) to the animal, responding on the left lever results in food reinforcement; if the monkey was given saline, responding on the right lever would be reinforced. This "interoceptive" discrimination is believed to be a model for subjective-like drug effects (e.g., Colpaert 1987). It is also an excellent model of CNS action of drugs. For the purposes of this review, we will highlight the role of drug discrimination as it relates to the scheduling of drugs by the FDA. For example, it is hypothesized that if the training drug is a drug of abuse (e.g., cocaine) and an unknown compound substitutes for cocaine, then that unknown drug has abuse liability. However, if the drug does not substitute for the training drug (in this case cocaine), it does not necessarily mean the drug lacks abuse liability. That is, drug discrimination informs us on one aspect of abuse liability, but that information alone is not enough for scheduling.

This review will focus on models of cocaine abuse, so a brief description of relevant drug discrimination findings will be described in this section. While

the theme of this chapter is the unique nature of using NHPs, in general, findings from cocaine discrimination studies in rodents are similar to the findings in NHPs. With that said, an inherent advantage of using NHPs is the ability to study many drugs over several years in the same subject. An additional advantage is the ability to combine several dependent variables to study the mechanisms of action of the training drug. For example, Howell and colleagues have shown that doses of cocaine that serve as discriminative stimuli elevate extracellular concentrations of DA and that other drugs that substitute for cocaine in drug discrimination also elevate concentrations of DA (Czoty et al 2000; Kimmel et al 2012). Combining measures of CNS action, using in vivo microdialysis and other brain imaging modalities (e.g., PET, MRI), with the study of behavior can best be conducted in NHPs. Such studies provide valuable information related to individual differences in vulnerability as well as treatment efficacy.

As it relates to scheduling of drugs, it is clear that elevating DA by blocking active reuptake will result in substitution to the cocaine-trained stimulus in many species (e.g., Spealman et al 1991; Terry et al 1994; Weed et al 1995). However, the FDA has not distinguished the importance of time course for this substitution in relation to scheduling. If one were developing a cocaine pharmacotherapy and compliance was important (which it is, of course), sharing cocaine-like discriminative stimulus effects, but with a slower onset and longer duration of action, may be beneficial. We will describe the evaluation of one drug in both models of abuse liability and attempt to evaluate how those behavioral data would impact scheduling of this drug (the drug was never evaluated by the FDA, so this is simply an exercise in testing the criteria).

The "test" drug we will describe is 2-ß-propanoyl-3-ß-(4-tolyl)-tropane (PTT), a long-acting DA transporter (DAT) blocker developed by Dr. Huw Davies (Davies et al 1993, 1994). PTT is approximately 20-times more potent than cocaine in binding at the DAT and approximately 50-times more potent at elevating DA than cocaine (Bennett et al 1995). These pharmacodynamic differences were apparent in behavioral studies. For example, in rats, PTT had a significantly longer duration of action compared to cocaine and was approximately 20-30 times more potent than cocaine in increasing locomotor activity (Porrino et al 1995). The first set of studies conducted in monkeys used male rhesus monkeys trained to discriminate 0.2 mg/kg cocaine from saline (Fig. 1). Administration of the training dose, 10 min before the start of the session, resulted in 100% cocaine-appropriate responding; administration of the drug vehicle resulted in 0% cocaine-appropriate responding (all responses were on the saline-associated lever). Other cocaine doses, tested 10 min before the start of the session, re-

sulted in dose-dependent increases in cocaine-appropriate responding (Fig. 1). When PTT was tested, doses resulted in 100% cocaine-appropriate responding when administered 1-5 hrs before the session. These data indicate (1) PTT has cocaine-like discriminative stimulus effects and therefore, may have abuse liability and (2) the duration of action was much longer than cocaine's effects; when tested 5 hrs after administration, the training dose of cocaine resulted in primarily saline-appropriate responding. These two observations will be critical to understanding the reinforcing effects of PTT, described in the next section.
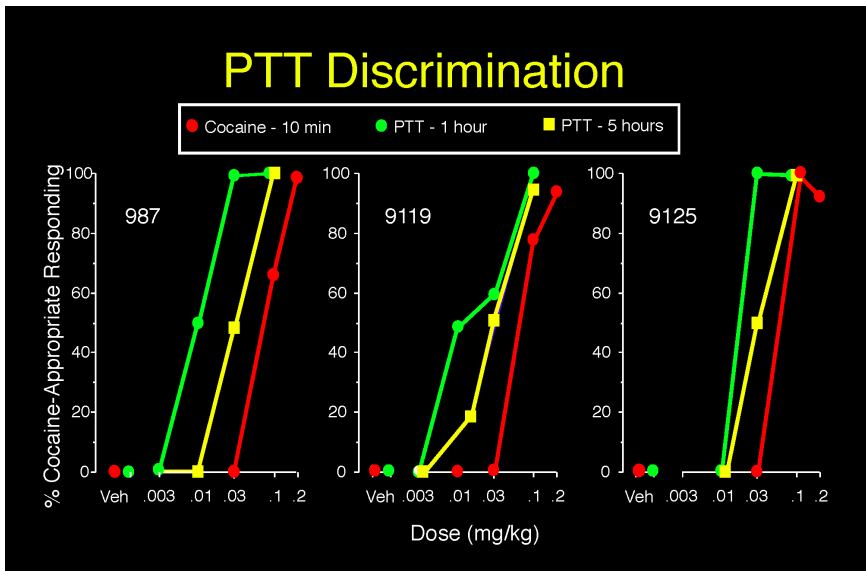
Figure 1:
The discriminative stimulus effects of cocaine in adult rhesus monkeys. Monkeys were trained to discriminate 0.2 mg/kg cocaine (i.m., 10-min pretreatment) from saline (0.5 ml/10 kg). Cocaine (red symbols) produced dose-dependent increases in cocaine-appropriate responding. Different doses of PTT, tested at 1 hour (green symbols) and 5 hours (yellow symbols) resulted in dose-dependent and complete substitution for the cocaine stimulus. Redrawn from Nader et al (1997).

3. Drug self-administration models

There is no animal model that is more predictive of human disease than drug self-administration models of abuse liability. The first demonstration that animals would press a lever to deliver a drug to themselves was by James Weeks in 1962 showing that rats implanted with an intravenous catheter would self-administer morphine. In 1964, Travis Thompson and Charles Schuster showed that rhesus monkeys would also self-administer intravenous morphine. In both of these pioneering studies, the animals were first made physically dependent on morphine and then given the opportunity to self-administer the drug to alleviate withdrawal (an example of negative reinforcement). In 1969, investigators at the University of Michigan (Denaeu, Yanigita and Seevers) showed that monkeys did not require physical dependence on morphine for the drug to function as a reinforcer and that other drugs that do not typically induce physical dependence (e.g., cocaine) would also function as reinforcers. It has since been shown that drugs will function as reinforcers by the same routes that humans abuse them, including intravenous, oral and inhalation (Griffith et al 1980).

There are two general types of studies conducted with the self-administration model: assessing the abuse liability of novel drugs and the evaluation of potential treatments for drug abuse (see Lile and Nader 2003). For this review, we will briefly examine both types of models. For abuse liability assessment, we will use PTT as an example of a novel compound and describe results from self-administration studies and how these data address issues related to FDA guidelines. For evaluation of potential treatments, we will describe how the schedule of reinforcement guides assessment of pharmacotherapies, as well as the influence of environmental variables. Finally, we will attempt to describe studies in which NHPs offer a unique advantage over other animal species when examining abuse potential and novel treatment compounds.

3.1 Initial assessments of the abuse liability of PTT

Most abuse liability studies use fixed-ratio (FR) schedules of reinforcement, in which drug presentation is contingent on a specific number of responses. For example, under an FR 10 schedule, the 10th response results in drug presentation. Less frequently used are fixed-interval (FI) schedules of reinforcement, in which drug presentation is contingent on a response occurring after a specified period of time has elapsed. For example, under an FI 1-min schedule, the first response after 1 minute results in drug presentation. An investigator may choose to use an FI schedule in cases in which the drug has substantial re-

sponse rate-decreasing effects. In that case, the primary dependent variable would be number of injections, rather than total responses or response rates. Irrespective of the schedule of reinforcement or the dependent variable (response rate or reinforcement frequency), the behavior is characterized as an inverted U-shaped function of dose (e.g., Weeks 1962; Pickens and Thompson 1968). Response-contingent injection of vehicle or very low doses of drug maintain low rates of behavior, with increases in responding occurring with increases in the unit drug dose up to some maximum or "peak"; further increases in available dose result in lower response rates or number of injections. Low doses to doses that result in peak responding constitute the "ascending limb" of dose-response curve, while the lower response rates with higher doses represent the "descending limb" of the curve. As reviewed elsewhere (e.g., Schuster and Thompson 1969; Johanson and Schuster 1981), there are several hypothesized reasons for ascending and descending limbs, including the former representing reinforcing effects in the absence of rate-decreasing drug effects, while the latter may be due to satiation or rate-decreasing effects (see also Zernig et al 2004). As highlighted below, with PTT as an example, complete dose-response curves are critical to interpreting the abuse potential of a drug.

According to the FDA, "abuse potential" implies that the drug has positive reinforcing effects, maintaining self-administration at higher rates than vehicle. The FDA does not specify the schedule of reinforcement necessary to evaluate whether a compound functions as a reinforcer. Because one premise of behavioral pharmacology is that the schedule of reinforcement influences the behavioral effects of drugs, we recommend that there should be specific guidelines as to the schedule conditions necessary for evaluating abuse liability. The descriptions below emphasize this point.

In our initial study with PTT (Nader et al 1997), rhesus monkeys were trained to self-administer cocaine under an FI 5-min schedule of reinforcement and responding was characterized as an inverted U-shaped function of cocaine dose. When different doses of PTT were substituted for cocaine, the response was not different from when saline was substituted (Nader et al 1997), indicating that PTT did not function as a reinforcer *under these conditions*. It is important to point out that PTT shared discriminative stimulus effects with cocaine, so although that apparent model of abuse liability indicated potential for abuse, self-administration suggested otherwise. However, when studied under an FR schedule of reinforcement, PTT self-administration was higher than vehicle responding, but significantly lower than behavior maintained by cocaine (Birmingham et al 1998; Lile et al 2000a,b). Thus, the same drug studied under different schedule conditions resulted in different conclusions related to reinforcing effects and perhaps abuse liability.

These results raise some issues not typically considered by the FDA. What is the significance of the fact that PTT administration resulted in cocaine-like discriminative stimulus effects, but in two models of self-administration (FI and FR) appeared to have lower reinforcing effects than cocaine? Should PTT be considered similar to or lower than a Schedule II compound, like cocaine? A further consideration in the FDA's evaluation of abuse liability is the rate of extinction during substitution studies. As shown in Fig. 2, when saline was substituted for cocaine, the response declined to extinction levels within 5 sessions. However, when PTT was substituted for cocaine, there were higher rates on the first session and it required more sessions to reach stable responding (Lile et al 2000a). While the mean rate of PTT responding was similar to saline after several days, PTT required more sessions to reach this point and this factors into scheduling considerations. However, PTT shares discriminative stimulus effects with cocaine, so it should not be a surprise that there are differences in responding early in the substitution process. Studies in NHPs, using drugs from multiple classes, have shown that reinforcing effects and discriminative stimulus effects are not necessarily providing similar information as it relates to abuse potential (Ator 2002; Martelle and Nader 2009). A final consideration involves examining self-administration in previously drug-naïve subjects. PTT did not function as a reinforcer in cocaine-naïve monkeys (Lile et al 2000a), yet that information is not one of the factors that the FDA considers as part of the scheduling process.
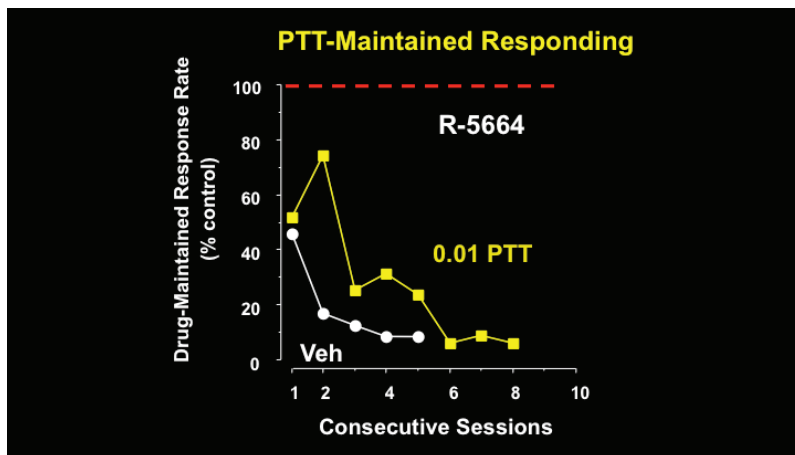

Figure 2:
Rate of responding following saline substitution (white symbols) or 0.01 mg/kg PTT (yellow symbols) in monkey R-5664. Data are shown as a percentage of baseline (0.03 mg/kg/injection cocaine). Redrawn from Lile et al (2000b).

3.2 Assessing the abuse liability of PTT using more complex schedules

The drug discrimination and self-administration studies using simple schedules of reinforcement provide initial indications of abuse liability, but the scheduling of novel compounds should involve models that allow for the direct comparison with known drugs of abuse. It has been suggested by several investigators that the use of more complex reinforcement schedules that assess measures of reinforcing strength or reinforcing efficacy provide a better indicator of a compound's abuse liability (Woolverton and Nader 1990; Banks and Negus 2012). The two most frequently used models are the progressive-ratio (PR) schedule and drug choice paradigms (either drug vs. drug or food vs. drug). For PR schedules, the number of responses required for a drug injection increase with each injection (Rowlett et al 1996; Stafford et al 1998) and the primary dependent variable is the final ratio completed, termed the break point, when no injections have been received after a specified period of time (termed the limited hold) or at the end of the session. The shape of the dose-response curve is an inverted U-shaped function and drugs can be compared on this measure of reinforcing strength by statistically examining differences in number of injections obtained at the peak break point (Richardson and Roberts 1996). For studies involving drug choice, the primary dependent variable is percentage of trials the drug is chosen. For drugs of abuse, the dose-response curve is represented as a monotonic increase in frequency of drug choice with increases in dose.

When studied under a PR schedule, PTT maintained responding higher than saline and break points varied as a function of dose as shown in Fig. 3. However, the peak break point for PTT was significantly lower than the break point for cocaine (Lile et al 2002). As it relates to the use of NHPs vs. other species, Roberts et al (1999) reported higher break points for PTT compared to cocaine in rats – an effect opposite to those reported in NHPs. These findings suggest that for some drug classes (e.g., long-acting DAT blockers), the choice of animal species will influence measures of abuse liability. Consistent with this hypothesis, another high-affinity DAT blocker developed by Huw Davies (WF-23), with an even longer duration of action than PTT, did not function as a reinforcer in NHPs (Lile et al 2000a), but had greater reinforcing strength than cocaine in rodents (Roberts et al 1999).
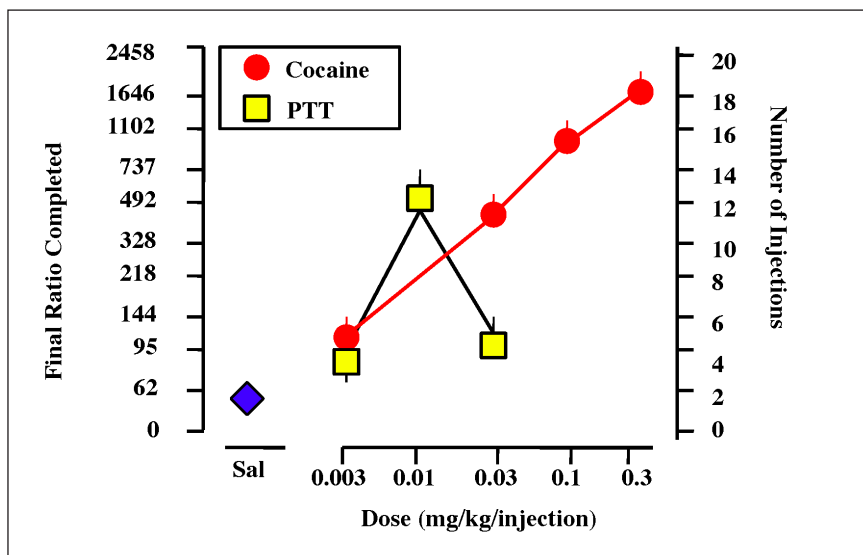
Figure 3:
The reinforcing strength of saline (blue symbol), cocaine (red symbols) and PTT (yellow symbols) as determined by responding under a progressive-ratio schedule of reinforcement. Left ordinate represents the final ratio completed; right ordinate indicates number of injections received per session. Each point represents the mean (± SEM) from four adult male rhesus monkeys. Redrawn from Lile et al (2002).

To summarize the self-administration findings in NHPs described above, PTT maintained lower rates of responding under FI, FR and PR schedules compared to cocaine. A final measure of abuse liability examined with PTT was to give monkeys the opportunity to choose between doses of cocaine and PTT under a concurrent drug-drug choice paradigm (Lile et al 2002). NHPs implanted with double-lumen catheters have been trained to choose between two drugs (e.g., Johanson and Schuster 1975; Woolverton and Johanson 1984), allowing for the most direct comparison of measures of reinforcing strength. There are several interesting comparisons, but two that will be highlighted here are (1) what happens to choice when the comparison is between doses of each drug that resulted in similar break points and (2) what happens when doses that resulted in peak break points (i.e., maximal reinforcing strength) are compared? Examining Fig. 3, the break point for 0.03 mg/kg cocaine and 0.01 mg/kg PTT were very similar. When monkeys were given a choice between these two doses, they choose 0.03 mg/kg cocaine on over 80% of the trials (Lile et al 2002). Interestingly, when a higher dose of PTT was available as an alternative to a high cocaine

dose (0.03 mg/kg PTT vs. 0.3 mg/kg cocaine), monkeys chose both drugs on approximately 50% of the trials completed, perhaps suggesting that PTT may have abuse liability similar to cocaine. What was possibly most important about these results was that even though choice was about 50% for PTT and cocaine, monkeys self-administered less cocaine than under baseline conditions – a nearly 90% reduction in total cocaine intake! This illustrates that while researchers focus on percent drug choice, other dependent variables should be considered in the profile of drug effects.

To summarize this section as it relates to scheduling of drugs, a few salient points should be made. Drug discrimination provides valuable information related to CNS mechanisms of action and time course, but without self-administration data cannot be used in assessing abuse liability. In self-administration studies, the schedule of reinforcement can impact measures of abuse liability and with long-acting DAT blockers there appear to be species differences. For purposes of scheduling, measures of reinforcing strength, especially PR schedules, are more appropriate than using simple schedules of reinforcement.

4. Use of NHP to evaluate treatments for drug abuse

A theme of this chapter has been to describe conditions in which the use of NHPs is advantageous over the use of other animal species when studying issues related to drug abuse. In addition to the studies described above for assessing abuse liability, the use of NHPs to examine potential treatment compounds has several advantages. As noted above, NHPs, compared to rodents, are more similar to humans in several aspects of neuroanatomy, neurophysiology and neuropharmacology. A second advantage is the ability to use the same monkeys over years, to allow for within-subject, longitudinal studies. Related to this last point, monkeys that self-administer drugs of abuse over years are a better model for evaluating treatment compounds than studies in which drug exposure is relatively short. When one considers that cocaine addicts may abuse the drug for decades before seeking treatment, the longer the history of cocaine exposure in animal models, the better the predictive validity. With regard to stimulants, there are currently no FDA-approved treatments (e.g., Haile and Kosten 2013; Heidbreder 2013), so identifying viable treatments is a goal of preclinical research. In this section, we will describe the use of two NHP models for evaluating drug treatments – PR schedules and concurrent schedules. There are excellent reviews on this topic (e.g., Johanson 1975; Mello and Negus 1996; Haney and Spealman 2008; Banks and Negus 2012).

4.1 Use of PR schedules in evaluating potential pharmacotherapies in NHP

The vast majority of previous and current research examining the effects of treatments on cocaine self-administration utilized procedures in which access to cocaine was limited to a maximum time and/or amount per day. We have combined chronic drug treatment with long access to cocaine under a PR schedule and have shown that cocaine self-administration is a stable and reliable baseline (e.g., Czoty et al 2006, 2010, 2013), allowing for the comparison of different drugs in the same subject. By comparing different drugs in the same subject, information related to individual differences can be garnered. This is an important point because not all treatment drugs are efficacious in all monkeys (or humans used in clinical trials), so understanding the phenotypic characteristics associated with treatment outcome is critical for developing customized treatments. For these studies, monkeys had the opportunity to earn their daily allotment of food by responding on one lever under an FR 50 schedule of reinforcement each morning. The pattern and amount of food consumed is an important dependent variable used to assess the side-effects profile of the drug treatment. Later in the day, monkeys had access to cocaine under a PR schedule in which cocaine was available for up to 20 hrs and until 2 hrs elapsed without an injection. Change in cocaine break point at each cocaine dose, was the important dependent variable for assessing treatment efficacy.

The overall strategy for examining putative pharmacotherapies is presented in Fig. 4. Amphetamine was chosen as an example for initial testing of the model because of previous preclinical and clinical studies suggesting efficacy as a cocaine pharmacotherapy (Grabowski et al 2001; Negus and Mello 2003a,b). Prior to the start of treatment, responding was maintained by a cocaine dose on the ascending limb of the dose-response curve. In order to better model the human condition in which the treatment seeker abstains from cocaine (at least initially) during the initiation of treatment, cocaine availability was terminated and a dose of an experimental drug, in this example, *d*-amphetamine, was administered intravenously at a rate of 0.4 ml/hr for six days (Fig. 5). On the seventh day of treatment, the monkey had the opportunity to self-administer cocaine in the absence of amphetamine treatment. On days 8 through 13, the same dose of *d*-amphetamine was again administered chronically in the absence of cocaine self-administration, followed by another self-administration session on day 14. If, at that point, no alteration in break point was observed, the dose of *d*-amphetamine was increased and the regimen started over. If an effect on cocaine self-administration was observed on day 14, treatment with that dose of *d*-amphetamine continued for an additional 16 days in the absence of cocaine
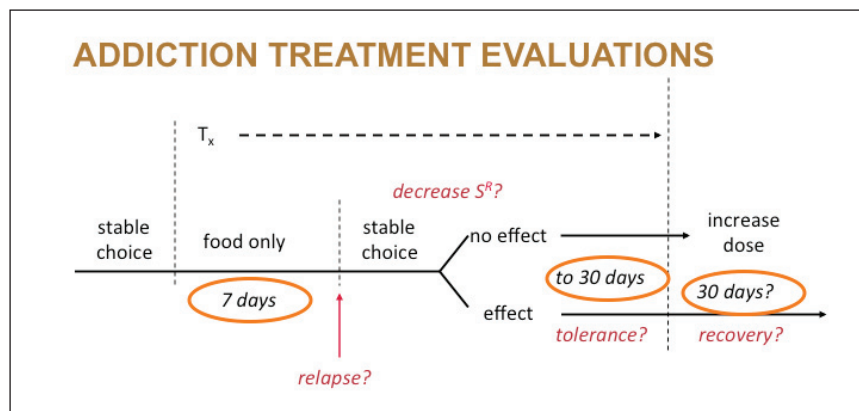
Figures 4:
Decision tree for evaluating potential pharmacotherapies in monkeys. During chronic treatment, monkeys respond for food presentation daily, while only self-administering cocaine once per week under a progressive-ratio schedule. See text for more details.

self-administration sessions to assess whether tolerance developed to the effects of the treatment drug on the reinforcing strength of cocaine. On day 30, a final self-administration session was conducted. If at that time no tolerance developed (i.e., the number of reinforcers earned under the PR schedule remained decreased from baseline), higher cocaine doses were evaluated every three days, with treatment continuing on intervening days, to see whether the effects of the treatment drug could be surmounted. If, on the other hand, tolerance developed to the effect of the treatment drug and the number of cocaine injections recovered to baseline values on day 30, treatment was discontinued, rather than increasing the treatment dose. The PR schedule was presented again 7 and 14 days later to examine whether a rebound increase in the reinforcing strength of cocaine occurred after discontinuation of treatment. A major strength of this design is the individualized nature of the treatment regimen. While all monkeys were tested with the same doses of *d*-amphetamine, different doses were effective in each monkey.
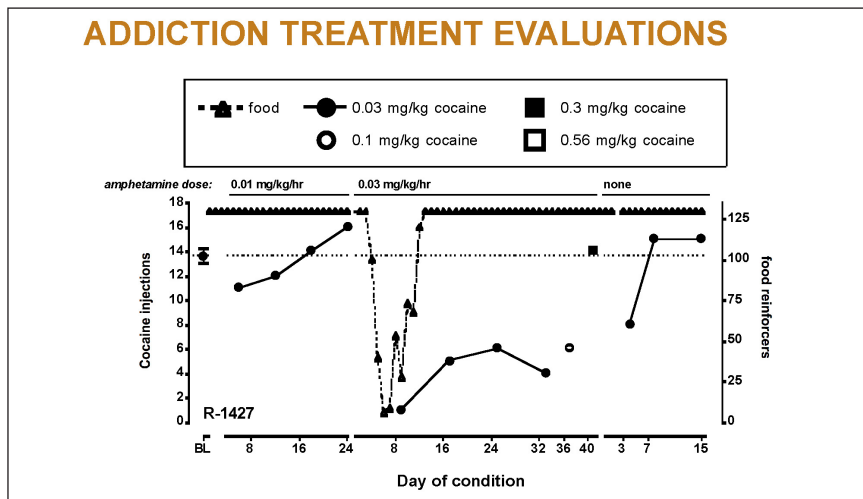
Figures 5:
The effects of chronic *d*-amphetamine treatment on food (triangles) and cocaine self-administration in rhesus monkey R-1427. Redrawn from Czoty et al (2011).

An illustrative example of data from one monkey is shown in Fig. 5 following chronic *d*-amphetamine treatment (Czoty et al 2011). Initially, monkey R-1427 was treated chronically with 0.01 mg/kg/hr *d*-amphetamine for 24 consecutive days, most of which were in the absence of cocaine. As seen in Fig. 5, *d*-amphetamine did not disrupt food-maintained responding (filled triangles), while the initial effect on 0.03 mg/kg cocaine self-administration was a slight decrease in break point to which tolerance developed. Increasing the *d*-amphetamine dose to 0.03 mg/kg/hr resulted in initial disruptions in food-maintained responding, but tolerance developed to those effects. Importantly, *d*-amphetamine decreased the break point for cocaine and tolerance did not develop to the effect on cocaine reinforcement (Fig. 5). Both findings represent characteristics of a potentially effective agonist therapy for cocaine abuse. These findings support the use of a DA agonist for the treatment of cocaine abuse but suggest that treatment should be continued (perhaps) indefinitely to maintain clinical efficacy.

4.2 Use of food-cocaine schedules in evaluating potential pharmacotherapies in NHP

It has been argued that the food-cocaine choice paradigm is the superior model with regard to assessing treatment efficacy (Banks and Negus 2012). Because addiction can be conceptualized as a choice disorder (Heyman 2009; Banks and Negus 2012), animal studies using drug vs. non-drug choice behavior may have the greatest face validity to the human condition and would most effectively facilitate translation of results to clinical settings (Haney and Spealman 2008; Hutsell et al 2015). In these studies, the non-drug alternative reinforcer (food pellets) is hypothesized to represent other potential reinforcers in the drug-abuser's environment, such as family, employment or other enjoyable activities. The main objective of treatment is to aid the individual in re-allocating behavior away from drug procurement and drug use to other more socially acceptable activities.

An example of stable choice performance is shown in Fig. 6 for an adult male rhesus monkey (see John et al 2015 for experimental details). For these studies, the food-drug choice paradigm utilized was one in which complete cocaine dose-response curves were determined in each session (Negus 2003). As shown in the Figure (top panel, black squares), when the alternative to food pellets was no injection or a low dose of cocaine, this monkey chose food exclusively. At higher cocaine doses, this monkey chose cocaine exclusively; at high cocaine doses, total trials completed were less than maximal (bottom panel, black squares). When saline was substituted for cocaine (white circles), the choice curve shifted downward (top panel) and total trials increased, representing more food choices (bottom panel) – this is the effect one would hope to see with a pharmacotherapy. The Figure also shows the effects of two DA receptor compounds, administered for 5 consecutive days, on cocaine-food choice. Treatment with the non-selective DA D2/D3 receptor antagonist eticlopride (orange symbols) resulted in a leftward shift in the cocaine dose-response curve (Fig. 6, top panel), up to doses that eliminated responding (Fig. 6, bottom panel). This suggests that at low doses, blockade of DA receptors increases the reinforcing potency of cocaine, a finding consistent with other preclinical food-cocaine choice studies (Woolverton and Balster 1981; Negus 2003; Czoty and Nader 2013). In contrast to these effects, when this monkey was tested with the novel DA D3-receptor selective partial agonist CAB 2-015 (blue symbols), developed by Dr. Amy Newman, the cocaine dose-response curve was shifted to the right, in a manner similar to what was observed when saline was self-administered. Interestingly, *d*-amphetamine also decreased cocaine choice under conditions similar to those described in this monkey (Negus 2003). Future studies are required to
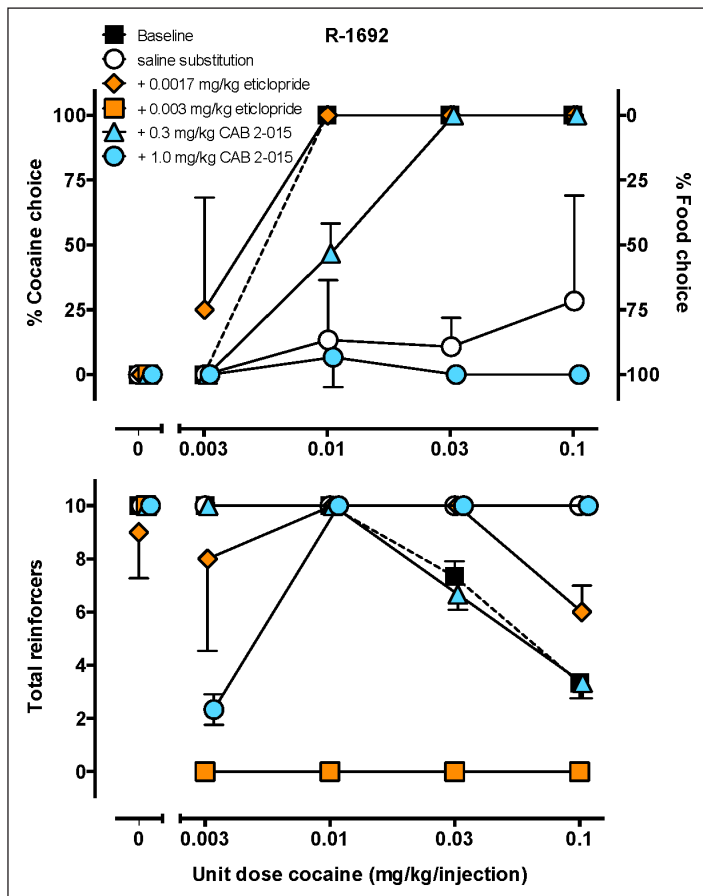
Figure 6:
The effects of chronic treatment with the dopamine D2/D3 receptor antagonist eticlo-pride (orange symbols) and the D3 receptor partial agonist CAB 2-015 (blue symbols) on cocaine vs. food choice (black squares) in rhesus monkey R-1692. Top panel represents percentage of the trials that cocaine was chosen over food. Bottom panels represent total trials completed out of a maximum of 10 trials per component. Each point represents the mean (± SD) of the last three sessions.

better understand how a DA D3 receptor partial agonist can produce effects on choice behavior similar to those of an indirect-acting DA agonist.

To summarize this section, the use of measures of reinforcing strength involving PR schedules and concurrent food-cocaine schedules allows for the most valid predictors of treatment efficacy. In both examples, there was a non-drug (food-reinforced) baseline on which to assess the direct effects of a potential treatment on behaviors not related to drug seeking. The use of NHPs is advantageous because of the ability to study treatments administered chronically over many months, to better model the human condition. Also, NHPs allow for the examination of within-subject effects of multiple treatment compounds and the study of individual differences in treatment efficacy.

5. Conclusions

The primary goal of this review was to highlight advantages for using NHPs in assessing abuse liability of drugs and in the evaluation of potential treatment compounds. It is now well established that drug addiction is a brain disease and that there are individual differences in terms of vulnerability and treatment efficacy. The use of group designs in which all animals receive the same treatment is not ideal for these types of studies. In addition to the similarity in brain neuroanatomy, neurochemistry and hormone concentrations, the use of NHPs allows for the study of behavioral and neurochemical phenotypes that would be predictive of the human condition. Treatment seekers will have different drug histories, family histories, stress levels, etc, which will certainly impact treatment outcome. The research described in this review point to the importance of studying individual differences and the advantages NHPs impart on the study of behavioral phenotypes. Rather than using a group design and discounting individual differences as "variable" or "equivocal", these differences should be studied in order to develop customized treatment strategies.

6. Acknowledgements

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Juvenile Toxicity Testing: Experience Using Nonhuman Primate Models

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Abstract

Toxicity testing and safety assessment in the juvenile nonhuman primate (NHP) is generally feasible but requires a number of specific considerations. A pivotal consideration is the choice of an appropriate experimental animal age both in terms of target organ maturation and in terms of animal supply. For the postnatal study of specific organ systems some recommendations have been proposed. Challenges can arise if neonatal or very young animals are required since transport of these animals without maternal presence is not permitted or very limited. Generating infant animals on the test site can be considered but is laborious and not very practical. On the other hand, a large battery of standard tests but also special test parameters are available to be used for assessing potential test item effects and to study specific organ functions. Examples for special testing are endocrine factors (e.g. adrenal steroids), imaging approaches, assessment of immune system functions, central nervous system (e.g. learning & memory test), bone development, etc. Also, various routes of administration are feasible including infusion dosing (e.g. intrathecal). In our experience, experimental protocols are very much tailored for purpose rather than using a standardized study design. Cases for experimental studies designs are provided. In general, once the age aspects have been solved, the conduct of a toxicity study in juvenile NHPs is not too different from that in older animals.

1. Introduction

Some guidances on juvenile safety assessment are available for consideration when developing compounds with desired or potential pediatric indication (FDA 2006, EMA 2008). Whilst these guidances are not harmonized with regard to the timing of the preclinical toxicity evaluation relative to the clinical studies, there is coherence in that juvenile toxicity testing should be considered if the available preclinical and clinical data are not sufficiently comprehensive to support the intended pediatric clinical trials. This has led to an increased

need for the conduct of safety assessment in juvenile animals across several species. Mostly these studies are performed using rodent models (rats and mice) but in a number of cases, NHP models have also been used. The choice of species is basically driven by the same considerations as for general safety assessment in that only relevant species should be used (Baldrick 2013, Barrow et al 2013, Leconte et al 2011, Morford et al 2011).

We have experienced an increased demand for the use of the NHP model in pediatric testing over the past years plus the occasional need to resort to non-standard routes of delivery (e.g. intrathecal) in these juvenile NHP studies. This chapter provides an overview of the experimental study designs and on the specifics of using juvenile NHPs for safety assessment. Also this chapter provides detailed information on intrathecal dosing of juvenile cynomolgus monkeys and a review of the standard and non-standard study parameters that have been used in these investigations. On a general note, although an attempt had been made to lay-out a "standardized" pediatric study design for cynomolgus monkeys (Chellman et al 2009), in reality essentially every single study used a case-by-case purpose-tailored experimental design.

2.   NHP species and availability considerations

The NHP model used was the cynomolgus monkey/long-tailed macaque (*Macaca fascicularis*) with the exception of a single study in which the common marmoset (*Callithrix jacchus*) had been used. No studies were conducted in rhesus monkeys (*Macaca mulatta*). This species distribution follows the general trend seen over the past years (at least for our laboratory) that the cynomolgus monkey is by far the predominant animals model followed the common marmoset, if NHP models are required during preclinical safety assessment.

An important difference between rodents and NHPs resides in the fact that time-mated NHPs are not commercially available and that the transport of neonatal/juvenile NHPs is strictly regulated depending on the animal age. It is therefore essential to distinguish between neonatal and juvenile toxicity studies for NHPs. At present, three animals supply options can be considered:

(a) To generate neonatal/juvenile animals at the test site. This would require a mating progamme with mature female NHPs, follow the animals throughout pregnancy until delivery and the initiate the toxicity testing in the newborn at a desired age. Essentially, this compares to the conduct of a pre- and postnatal development (PPND) study but without dosing the maternal

animals and would require approximately 16 months until groups of infants would be available for testing. Potential caveats are the primate-inherent pre- and postnatal losses and the lack of control of gender distribution in the newborn. Not withstanding that, to date, our laboratory has used this approach in two instances.

(b) Around certain ages, infant NHPs can be transported but only together with the maternal animal. For example, juvenile and maternal animals could be imported together along with their mother at an approximate infant age of 5.5 months. The caveats for this approach are a very difficult animal supply scenario and the question about the fate of the maternal animals after termination of the infant study phase – it would not be considered ethical to terminate the maternal animals, in which case another strategy must be in place. Our laboratory has not used the approach of co-import of maternal and infant animals to date.

(c) Import juvenile animals without maternal animals. Whilst there are some differences among different suppliers, in general NHPs need to be older than 12 months in order to be able to transport the animals. Occasionally 9-12 months were found to be acceptable. For our laboratory, this has been the typical approach of animal supply for NHP juvenile toxicity evaluation and permits gender control. Figure 1 provides an exemplary scheme and

Figure 1:
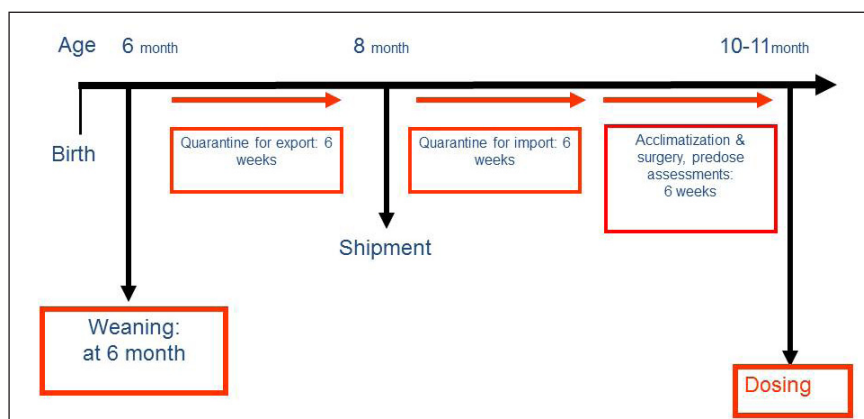Exemplary scheme and timelines for a NHP juvenile toxicity study in cynomolgus monkeys with dose initiation at approximately 10-11 months of age. A commitment to the study is required 4-5 months prior to study start in order to implement this scenario. Essentially, the animals are weaned at the age of 6 months but then are housed and quarantined (export and import) in groups until transport.

timelines for a NHP juvenile toxicity study with dose initiation at approximately 10-11 months of age. It is important to note that for animal welfare considerations and from a logistics perspective, a commitment to the study is required 4-5 months prior to study start in order to implement this scenario. Essentially, the animals are weaned at the age of 6 months but then are housed and quarantined (export and import) in groups, and also are socially housed thereafter with group sizes depending on the study design.

3. Terminology of postnatal development and age aspects: NHP vs human

The timing of the various postnatal phases is obviously different across species (rodents, dog, minipig, primates) and these differences need to be considered for study planning and/if the test item targets a particular organ system (Baldrick 2010 & 2013). For NHPs, there is a lack of accepted and consistent definition of the various postnatal development phases. However, efforts have been made to provide this information for the cynomolgus monkey (Morford et al 2011, Weinbauer et al 2011a) and this information is presented in Table 1. In this book, the chapter by Martin describes an approach on how to use the specific postnatal timing and maturation information for the conduct/waiver of dedicated juvenile toxicity evaluation in the NHP model.

Table 1:
Postnatal phase definitions in the cynomolgus monkey and comparison to rhesus monkey and human

	Cynomolgus monkey	Rhesus monkey	Human
Newborn	24 hr postnatal	24 hr postnatal	At term
Neonate	0–4 months	0–1 month	0–1 month
Infant	Up to 8 months	1–12 months	1–24 months
Juvenile	Up to 36 months	12–24 months	Not defined
Adolescent	3–5 years	2–4 years	12–16/18 years
Young adult	5–7 years	4–8 years	16/18–20 years
Adult	> 7 years	8–15 years	>20–25 years

Based upon Morford et al (2011) and Weinbauer et al (2011a). Note that adulthood is defined by epiphyseal closure. Hence, the term young adult versus adult appears somewhat arbitrary.

The knowledge about the postnatal timing of organ system development is critical in order to select the appropriate animal species development phase in order to be representative of the human target development period/organ. For the above species including human, age conversion factors have been provided earlier, initially starting with rat and human (Buelke-Sam 2003). One point of reference for comparing postnatal development across species has been the timing of sexual maturation, and for cynomolgus monkeys a factor of 3-5 has been derived (Morford et al 2011) and for practical reasons, a factor of 4 is currently being used for translating NHP animal age into the clinical pediatric situation. Table 2 provides a comparison of the postnatal maturation of selected organ systems between cynomolgus monkeys, rhesus monkeys and human and is based upon Martin and Weinbauer (2010).

Table 2:
Postnatal timing of selected organ system developments/recommended minimum ages

Organ system	Cynomolgus	Human	Recommended minimum study age
Skeletal**			
Ossification centers	0–6 months	6 months - 12 years	12 months
Epiphyseal closure	5–9 years	11–20 years	not applicable
Immune			
Spleen/lymph node	Birth	Birth	not applicable
Immunocompetence	0–3 months	0–3 months	3 months
Reproductive	3–6 years	8–14 years	not applicable
Cardiovascular	Birth	Birth	not applicable
Renal	Birth	12 months	12 months
(Central) Nervous			
Locomotion	7 weeks	9–13 months	2 months
Fine motor/dexterity	6 months	1.5–13 years	6 months
Sensory/reflexes	0–12 months	0–12 months	12 months
Cognition	3 weeks–3 years	1 months–9 years	3 years
Communication	0–12 months	0–24 months	12 months
**Pulmonary	Birth	2–8 years	not applicable
Digestive	0–8 months	0–24 months	8 months

Based upon Table 1 from Martin & Weinbauer (2010) and Morford et al (2011). The right column represents the recommendation for the minimal age of macaques for studying potential organ system effects. ** denotes systems with major functional differences compared to human postnatal development. Bone age at birth is far more advanced in macaques than in humans. For the skeletal system, differences also depend on which bone is being studied.

4. Study design and parameter selection for NHP juvenile toxicity assessment

4.1 General considerations

Standard considerations have been provided earlier on exemplified experimental design proposals for juvenile NHP studies (Chellman et al 2009, Morford et al 2011). General safety assessment parameters such as clinical signs, body weight, food consumption, clinical pathology, ophthalmology, cardiovascular function, immuntoxicology, bone and skeletal parameters, neurobehaviour and histopathology can normally be tested conveniently. Certain limits maybe imposed by the availability of blood volumes for toxicokinetic (TK) testing, anti-drug antibody (ADA) analysis in case of biopharmaceuticals or special immunotoxicology testing (e.g. natural killer cell activity). Table 3 provides a synopsis of the general and special parameters available for the assessment of postnatal development and juvenile toxicity assessment in the cynomolgus monkey model.

During 2004-2013, our laboratory has initiated 15 juvenile toxicity studies in the NHP model. In a third of these studies, the test item was a biopharmaceutical. A synopsis of selected parameters from these studies is provided in Table 4. Fourteen studies were conducted in cynomolgus monkeys and one study in the common marmoset. The study age range for the cynomolgus monkey was from one month till 2.5 years of age but typically animals were 9-15 months at study start. For the marmoset, animals were dosed during 2-4 days of age. Animal numbers per study were highly variable and ranged from 3-50 animals. Equally, study duration was very variable and ranged from one day to 53 weeks of dosing plus a 26 weeks recovery period. The routes of administration were oral, subcutaneous, intravenous and intrathecal. The spectrum of parameters that were assessed is summarized in Table 3.

4.2 Learning and memory testing

Learning ability in the cynomolgus monkeys is typically assessed by use of the Wisconsin General Testing Apparatus (WGTA) and is based upon double object discrimination. The test requires that animals are being trained and – equally important – that the experimenter is also well trained and executes the testing sequences as consistently as possible. It is also essential, that the environmental conditions are kept strictly constant between the various testing occasions in order to obtain reproducible and relevant data. The test comprises a habitua-

Table 3:
Selection of parameters available for a toxicity study in juvenile cynomolgus monkeys

Clinical signs	As appropriate
Body weight	As appropriate
Food consumption	As appropriate
Clinical pathology parameters (as applicable)	Hematology, serum chemistry, coagulation and/or urinalysis; time points pending on blood volume considerations
Special assessments (as applicable)	Toxicokinetics: after first and last dose and/or recovery (biopharmaceuticals) Ophthalmology: slit lamp biomicroscopy and indirect Ophthalmoscopy Cardiovascular: heart rate, blood pressure and/or ECGs Immunology: immunophenotyping, immunoglobulins, TDAR (e.g., KLH) assay, NK cell assay, cytokines, lymphocyte proliferation Bone maturation: radiographic evaluation of long bone. Quantitative measures of bone mineral content and density (DEXA, pQCT), serum markers of bone metabolism (NTx, CTx, osteocalcin) Neurobehavioral testing: available (e.g. learning and memory) but options more limited than rodents Imaging: CT, MRI, PET
Terminal procedures	Complete necropsy of all animals, including gross pathology and organ weights Full tissue collection and histopathologic evaluation Immunohistochemistry possible

TDAR, T cell-dependent antibody response; NK, natural killer cell; KLH, keyhole limpet hemocyanin; DEXA, dual energy X-ray absorptiometry; pQCT, peripheral quantitative computed tomography. Based upon Morford et al (2011).

Table 4:
Covance juvenile NHP toxicity study experience

Period	2004 – 2013
No studies	15
Species	cynomolgus/marmoset
Age	1 m – 2.5 y (9-15 m; d 2-4 - marmoset)
Origin	asian, mauritian (cynomolgus)
Size	3 – 50 animals
Duration	1 d – 53 w/26 w rec
ROA	po, iv, sc, it
Parameters	general, clinical pathology, behaviour, learning & memory CSF, bone, imaging, immune system, DART

tion phase and a learning phase plus reversal learning/memory testing. Once the animal achieves 80% or more correct choices on two consecutive days, the definitive learning test is being performed.

Equally important, animals need to be about six months old in order to be eligible for successful training for this type of test. In fact, as per ICH S6(R1) (2011), this test is not recommended as it would prolong the duration of the postnatal observation phase for infants in ePPND studies. Because of several sources of variation inherent to the test, it has been estimated that the statistical power of this test in NHPs is rather poor and large animals numbers are needed in order to detect significant differences in learning abilities (Cappon et al 2012). On the other hand, others have reported favourable experiences using this test in the cynomolgus monkey model (Makori et al 2013). We observed recently in our laboratory rather good performance success using this test in juvenile toxicity studies such that at a group size of eight animals a statistical power of 0.80 was achieved for detecting a 50% difference (Rose et al 2015).

4.3 Physical, neurological and neurobehavioural examinations

Physical and neurologic examinations can be performed in unsedated animals. Physical examinations included abdominal palpation, eye, ear and nose examination, body temperature, heart, and lung auscultation. Neurologic examinations include general sensomotorical sensomotory aspects, cerebral reflexes (pupillary, orbicularis oculi, cornea) and spinal reflexes (patellar, anal). The following additional tests can also be performed: Patellar reflex (the presence or absence of a quick extension of a hind leg following the tapping of the patellar ligament with a hard object), foot grip reflex (a rod is placed under the foot and the animal should grip this rod).

For neurobehavioral investigations, animals are observed using a standard observation battery, and in their home cage, thus permitting the assessment of both peripheral and central nervous systems activities. This approach represents a modified version of the primary observation test described by Irwin for detecting neurological and behavioral changes in mice Irwin (1968). Table 5 provides a synopsis of the parameters that are being used in the juvenile cynomolgus monkey.

Table 5:
Parameters applied during a modified "Irwin" test in the cynomolgus monkey

Parameters	Explanation
Checking Vocalization	Mouth partially closed with abdominal contractions
Communicative Vocalization	Mouth open, often with increasing volume
Alertness	Animal response to technician approaching cage
Stereotypy	Side to side head movement, hallucinatory visual tracking, circling behavior, retropulsion, repeated scratching, repeated biting of cage, repeated licking of cage
Grooming Incidence	
Aggression – Offensive	Animal moves to front of cage, bears teeth and possibly vocalizes
Aggression – Defensive	Animal moves to back of cage, bears teeth and possibly vocalizes
Posture	
Balance/Coordination	
Locomotor Activity	Animal activity level within cage
Tremor	Involuntary muscle contractions causing oscillatory movement of body, head or limbs
Convulsion/Twitches/Jerks	Involuntary muscle contractions causing animal to abruptly jerk or twitch
Reaction of Accustic Stimuli	Animal reaction to lip smack
Novel Visual Stimuli	Animal tracks movement of an object in front of cage
Muscle Tone	Assess by palpation of limbs and abdomen
Grip Strength	Assess the grip strength of the monkey during removal of the animal from the cage by observing the monkey's ability to grip a part of the cage
Body Temperature	

5. Case studies in the juvenile NHP model

5.1 Case study 1

This study evaluated the endocrine effects of a HIV-NNRTI (van Velsen et al 2011). During preclinical testing in mice, rats and dogs, effects on endocrine adrenal functions were noted such that owing to partial inhibition of cytochrome P450 21-hydroxylase, decreased corticosterone and cortisol levels and

increased progesterone and 17alpha-hydroxyprogesterone levels were encountered. The effects on the adrenal functions were more pronounced that the effects on gonadal functions. Whilst no effects could be observed in rat ovaries, treatment of immature for about one months indicated signs of early genital and ovarian maturation and a relation to administration of HIV-NNRTI was conceivable. Against this background, a study was designed in immature female cynomolgus monkey. The NHP model was chosen since a significant androgenic adrenal pathway is present and because of the similarity of the adrenal gland physiology, the androgenic pathway, ovaries and estrogenic cycling. Immature animals aged 12-24 months with confirmed absence of ovarian cyclicity were selected and were dosed orally for eight weeks thus covering the duration of two complete ovarian cycles (Weinbauer et al 2008).

A plethora of parameters was studies in order to screen for premature activation of ovarian and adrenal functions. The screening comprised daily vaginal swabs for cycle detection, ACTH stimulations tests at five timepoints, determination at ten timepoints of endocrine factors (progesterone, 17alpha-hydroxyprogesterone, estradiol, LH, androstenedione, cortisol, DHEA, ACTH) monitoring of clinical pathology parameters and necropsy with complete macroscopic and microscopic examinations. The animals tolerated treatments and interventions well without noticeable clinical signs. Some minor effects on endocrinology (increased progesterone and 17alpha-hydroxyprogesterone but reduction of androstenedione and estradiol) whilst no effects were observed on cortisol and baseline ACTH. The latter observations matched those available from Phase III clinical trials. In contrast to dogs, no evidence was obtained for effects on the estrous cycle, ovarian histology or plasma LH levels, suggesting that the test item effects were limited to the adrenal without early maturation of the ovaries or the female genital tract in the NHP model. It was considered that these findings provided assurance that the test item will not have effects on the ovaries of genital tract in pediatric patients.

5.2   Case study 2

This study aimed at developing a strategy employing physiologically based pharmacokinetic modelling to investigate the intravenous use of the neuraminidase inhibitor oseltamivir and its metabolite oseltamivir carboxylate in infants and neonates with influenza (Parrott et al 2011). For modelling purposes, plasma and liver concentrations in adult and newborn marmosets after intravenous and oral dosing were obtained, and simulations with human model and data were undertaken. Specifically, female animals from the breeding colony were

allowed to litter and the neonates were kept with the parents for 2-4 days at which age the animals received single oral or intravenous dosing. Four animals per group were used and in-life data comprised clinical signs, body weights and blood collections for pharmacokinetic analysis.

The work concluded that the simulations in one-year-olds and neonates were in reasonable agreement with published results for oral doses (Parrott et al 2011). Importantly, neonates survived dosing and repeated blood collections and the parent animals tolerated well the repeated handling of neonates. This study principally demonstrated the feasibility of dosing neonatal NHPs at least under the circumstances of the chosen experimental conditions.

5.3   Case study 3

During advanced preclinical testing of monoclonal antibodies, enhanced PPND studies are being conducted in the NHP model i.e. the cynomolgus monkey (Weinbauer et al 2011b & 2013). Owing to specific IgG transport mechanisms in primates, the late fetus is massively exposed to the monoclonal antibody such that even at and after birth neonatal and infant animal may demonstrate relevant exposure for some months (Bowman et al 2013, chapter by Martin 2015 in this book). Therefore, early postnatal exposure during the conduct of an enhanced PPND study has been used to derive data and information for consideration of human pediatric trials. Also, infants from ePPND studies can be allocated towards specific juvenile toxicity assessments. In this particular case study, at the age of six months, a group of infants from a PPND study was maintained with their cage mates, but underwent dosing for about one month in order to address specific pediatric safety concerns. Quite a number of investigations were applied including clinical signs, body weight, clinical pathology, echocardiography and advanced in-vivo imaging. In all cases, the maternal animals tolerated repeated handling of infants, and the infants were already familiar with some procedures and handling from the previous ePPND study postnatal phase. Hence, (enhanced) PPND studies also provide various opportunities to address pediatric concerns in a relevant animal model.

6. Special considerations for central nervous system (CNS) targets in juvenile NHPs

6.1 Development of CNS administered biologics

In terms of drug delivery and administration, crossing the blood-brain barrier is often an important need in these days pharmaceutical drug development (Turner 2003). A thorough modelling of the blood-brain barrier in context with drug discovery and development has been outlined by Cecchelli (2007). For a pediatric target population, often bolus or continued intrathecal drug delivery is required in preclinical programs using juvenile cynomolgus monkeys (Felice 2011). In our experience assessment of juvenile toxicity within this species represents an emerging field, requiring established techniques for 12 month or younger monkeys.

Intrathecal delivery by spinal needle administration in sedated juvenile cynomolgus monkeys or with an implanted port-catheter system have successfully been conducted up to a duration of 53 weeks. At start of dosing the animals were approximately 10 to 11 months of age and weighed 1.0 to 2.0 kg for both genders. The evaluated endpoints included clinical observations (including postdose observations on dosing days), body weight, estimated food consumption, physical and neurologic examinations, test for learning ability, neurobehavioral observations (modified Irvine test), electroencephalography, ophthalmic examinations, determination of bone mineral density and bone mineral content, determination of femur length by X-ray, clinical pathology evaluations including urine analysis, CSF evaluations (clinical chemistry and cell count) and T cell-dependent antibody response (TDAR). Furthermore, perfusion necropsy, spinal cord brain trimming and pathology were conducted (Butt 2011).

6.2 Dosing by intrathecal spinal needle

Lumbar intrathecal administration of juvenile cynomolgus monkeys is feasible for up to 53 weeks. A dose volume of 0.75 mL – 2.0 mL of test item formulation, followed by 0.25 mL of a CSF can be used for each injection, if the approximate dosing volume of CSF is withdrawn in forefront. Best practice is to conduct a micro incision of the skin using a 20G needle before introduction of the spinal needle. Dosing is conducted with a Pencan® Paed pencil-point needles for pediatric use, 25G, 50 or 25 mm, B. Braun Melsungen AG, Melsungen, Germany (Figure 2). Using this dose route, administration intrathecally is being conducted via lumbar puncture at level L3-L4 and a slow manually

Figure 2
Special spinal needle, sturdy tip design, tip design allows for minimal intrathecal penetration depth and avoidance of incomplete blocks, Transparent needle hub, Color-coded stylet hub (Pencan® Paed pencil-point needles for pediatric use, 25G, 50 or 25 mm, B. Braun Melsungen AG, Melsungen, Germany).

infusion over 1 minute injection to fasted anesthetized animals (Ketamin and Domitor; Antisedan used as antidote). Doses can be administered at level L5-L6 as an alternative if the intrathecal space was not assessed at level L3-L4. Bepanthen® aseptic wound ointment (contains chlorhexidine and dexpanthenol; PZN 1987824) was applied immediately after each dosing.

6.3   Dosing via port-catheter

Lumbar intrathecal administration of juvenile cynomolgus monkeys is feasible for up to 53 weeks when using a surgically implanted port catheter system. Surgical implantation of the port catheter system is conducted approximately 3-4 weeks prior study start. A MID-LOVOL ports (titanium, low-volume) for intrathecal dosing (30 µL dead space) from Instech Laboratories, Inc. (Figure 3) has been used together with a 3FR intrathecal catheter (polyurethane), with at least 3 holes at the end and rounded tip (Instech Laboratories, Inc.). Pre-surgery (min. 0.5 hours before surgery) the animals receive Flunixin (Finadyne) 0.5 to 1.0 mg/kg. Then the animal receive i.m. Ketamin hydrochlorid 5 mg/kg and 0.06 mg/kg Medetomidin (Domitor) for sedation of the approximately 25 minute surgery, followed by shaving the back side of the animal (occipital to lumbal). The area of cutting is desinfected with an idol solution (e.g. Betaisodona solution) and a 18G Touhy or spinal needle was inserted between bones L2 und L5. The polyamide catheter (900 mmlengths require) is inserted through the needle in the subarachnoid space (length approximately 6 cm). Now a small pocket is prepared and the needle is taken out. Below the shoulder blade the
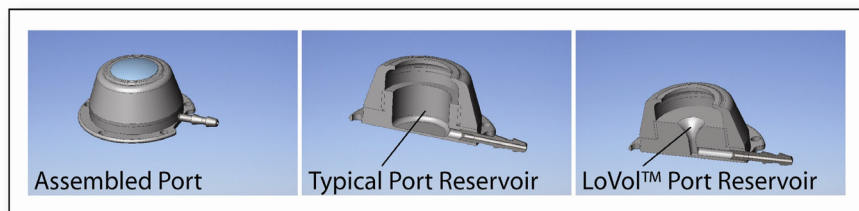
Figure 3:
MID-LOVOL ports (Titanium, low-volume) for intrathecal dosing (30 µL dead space) from Instech Laboratories, Inc.

Figure 4:
Lateral view of a cynomolgus monkey with implanted lumbar port catheter system.

port is inserted subcutaneously. The catheter is tunnelled with the help of a tunnel needle from caudal to cranial. At the lumbar catheter entry position a small lope was made and the catheter was fixed with tissue glue. The lumbar cut was closed with Vicryl. Now the catheter was connected to the port (Figure 4). After closure of the thorax pocket and the tissue cut, the system was checked (this can be done through the port with a Huber-, Gripper or SFN- needle using approximately 200 µL sterile 0.9% NaCl Solution or artificial CSF) for free flow and also using x-ray technology (with contrast medium). Post surgery the animal received an antibiotic (e.g. Veracin) and an analgesic with Flunixin (e.g. Finadyne) for 2–5 days. A wash out and recovery period, together with enriched

feeding (as a slight weight loss was anticipated) is routinely scheduled prior to the start of first dosing. All doses were administered as a slow bolus infusion using a calibrated syringe pump. The dose (0.6 ml + 0.7 mL flush of PBS were given over approximately eleven minutes). At dosing, the area around the subcutaneous access port was clipped free of hair if necessary. The area over the port was desinfected and was accessed with a needle attached to a 1 mL syringe for withdrawal of fluid in the dead space (approximately 0.4 mL).

Necropsy confirmed the placement of the catheter tips at T11/12. There were no adverse effects noted at the site of the catheter in the intrathecal space and in particular, no evidence of pronounced reactions at the catheter tips. Microscopically, slight to minimal morphologic changes at the catheter tip were noted with this technique. Tissue reactions (Figures 5a and b) were consistent with those reported to occur with the placement of intrathecal catheters in multiple species (Butt 2011). Changes at the catheter tip included slight to minimal fibrosis, adhesion to the overlying dura, and slight compression of the spinal cord (no cord damage).
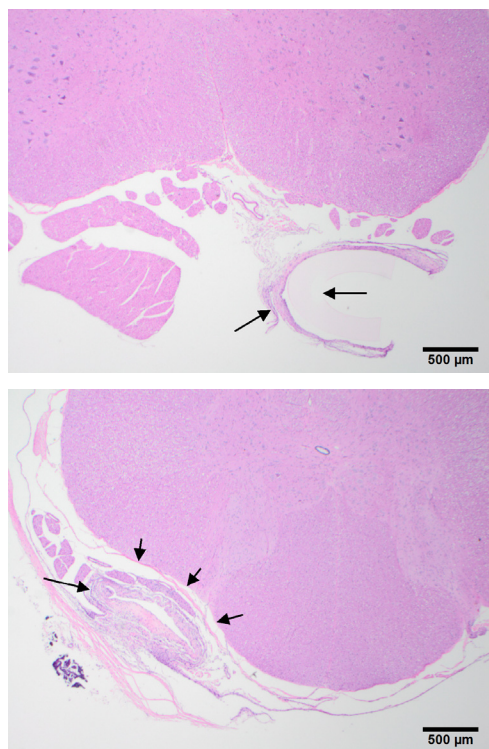
Figure 5:
(a) Spinal cord catheter track. The arrows indicate the catheter track, with the spinal cord being above the catheter. There is no cord compression visible and no infiltrates of inflammatory cells (Butt 2011); (b) Spinal cord catheter track. The arrows indicate the catheter track. There is a slight compression of the adjacent cord, but this is not associated with any adverse changes in the spinal cord. Compression is a commonly observed finding in the spinal cord adjacent to an intrathecal catheter. The reaction is limited to a thin rim of connective tissue (Butt 2011).

7. Conclusions

When planning and conducting juvenile toxicity studies in the NHP model, the following considerations are provided:

- Define age of clinical population/target organ system
- Define whether neonatal or juvenile animals are needed
- Age conversion factor of 4 is typically used
- Animals at juvenile age usually sufficient
- Studying neonatal animals is feasible but represents exceptional cases
- Juvenile toxicity evaluation can be incorporated into ePPND studies

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Considerations for the Development of Monoclonal Antibodies for Pediatric Indications: Using a Weight of Evidence Approach as an Alternative to Juvenile Toxicity Testing in NHP

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Abstract

Nonclinical juvenile toxicity studies may need to be considered to support the use of therapeutic monoclonal antibodies in pediatric patients. If needed they are generally conducted in rodents with the non-human primate (NHP) being used only when rodents are not pharmacologically relevant species. At birth NHPs are more developmentally advanced or as advanced as humans. For many of those organ systems that are not fully developed the most rapid period of postnatal development is within the first 3–6-months with continuation through to maturation, but at a slower rate. When considering the need to conduct juvenile toxicity studies the organ system of concern, the timing of greatest susceptibility and the type of molecule need to be carefully considered.

If sufficient information is available from developmental toxicity studies and/or general toxicities in young adults, additional studies in juvenile animals may not be need.

1. Introduction and regulatory considerations

Many drugs that are initially developed to treat adult patients have also been shown to be beneficial in pediatric patients. In addition some drugs are developed specifically to treat diseases in children. However, there is currently no harmonized regulatory guidance on the need for non-clinical studies in juvenile animals to support the use of these drugs in children. The United States Food and Drug Administration (FDA 2006) and the European Medicines Agency (EMA 2008) have issued regional guidances regarding the need for juvenile toxicity testing in animals to support pediatric indications. Although there are some differences in these regional guidances, especially with regards to the timing of the studies relative to clinical development, both guidances suggest that

juvenile animal toxicity studies may need to be considered when existing animal and human data are insufficient to support the planned pediatric studies. If a study is warranted, one relevant species, preferably rodent, is generally considered adequate. This recommendation is most applicable to small molecule drugs that can be tested in rodent species. The guidances further indicate that a non-rodent species may be appropriate when scientifically justified.

2. Factors to consider for juvenile toxicity testing

A number of factors need to be considered, irrespective of species, when deciding whether a juvenile toxicity study is warranted. Firstly, is the immature animal more sensitive than the mature animal with regards to the specific organ system of concern? Of particular importance is whether new toxicities are likely to be observed in immature animals with postnatal dosing that had not already been observed following exposure in pre and post natal development studies when the developing system may have been at its most sensitive. Differences in absorption, distribution, metabolism, and excretion in juvenile versus adult animals may need to be considered, although this is generally more of a concern for small molecule drugs than for mAbs. This is because mAbs are generally administered systemically, distributed mostly within the blood compartment, catabolized to peptides and amino acids that are reused by the body and are not metabolized by the liver enzymes (Vugmeyster et al 2012). With regards to pharmacodynamics the expression of the target during development may need to be considered. The age of the patient population also needs to be considered; for example pre-term infants and neonates may be considerably more sensitive than adolescents with regards to particular organ systems.

3. The use of NHPs for juvenile toxicity testing

Many mAbs and other biopharmaceuticals are designed to specifically target human antigens. As such, they frequently will not bind to the analogous target in standard toxicology species (rodents and dogs) and the non-human primate (NHP) may be the only pharmacologically relevant species for all of the non-clinical safety testing. For these molecules the non-clinical safety program may be limited to 1- to 6-month general toxicity studies in young or mature monkeys, usually cynomolgus macaques, and a developmental toxicity study in the same species. For mAbs intended to treat chronic disease indications, the preferred developmental study design is an enhanced pre and postnatal (ePPND) study in which pregnant monkeys are dosed from the beginning of organogen-

esis through parturition with examination of the infants for morphological and functional development (Chellman et al 2009; Stewart 2009; Weinbauer et al 2013).

NHPs are not routinely used for juvenile toxicity testing. Consequently there are no standard study designs for NHP juvenile toxicity studies but there are a number of endpoints that can be measured (Chellman et al 2009; Morford et al 2011, Makori et al 2013). If a NHP juvenile toxicity study is deemed to be necessary then each study needs to be designed based upon the specific concern being addressed.

4. Exposure to mAb during a pre- and post-natal developmental study

Numerous macaque studies have shown that for biopharmaceuticals containing the Fc portion of IgG (mAbs and Fc fusion proteins) the ePPND study design may result in considerable fetal exposure (end of organogenesis through parturition) and that the mAb may persist in the infant serum for a number of months post-birth (Martin and Weinbauer 2010; Bowman et al 2013). The fetal and neonatal exposure of Fc containing biopharmaceuticals (and albumin also to some extent) is a consequence of specific active transport via the placental FcRn receptor (Kuo et al 2010) and would not be expected to occur for other types of biopharmaceutical proteins. In macaques the concentration of the mAb in the fetal serum relative to that of the dams increases with gestational age such that at the time of birth the neonate may have exposure that is equal to or exceeds that of the mothers (Figure 1). Consequently if the dosing in the dams produces serum levels that are at least 10-fold in excess of the clinical exposure then the exposure in the fetus/neonate at the time of birth may also be in excess of 10-fold the clinical exposure. Fetal mAb exposure at the beginning of the fetal period will be lower than at parturition but may be as high as 1% of the maternal concentration (Bowman et al 2013). This may be sufficient to be within the pharmacological range for the molecule under investigation.

In humans and NHPs IgG antibodies do not appear to be transferred from mother to infant in the milk (other than local exposure to the gastrointestinal tract) and therefore the serum concentrations of antibody in the infant decreases after birth as the mAb is cleared from the serum (Martin and Weinbauer 2010). Since human mAbs can have half-lives of many weeks in monkeys and the monkeys are dosed at high multiples of the clinical dosing the monkeys may have pharmacologically relevant concentrations of the mAb in their serum for many months post-birth (Figure 1).
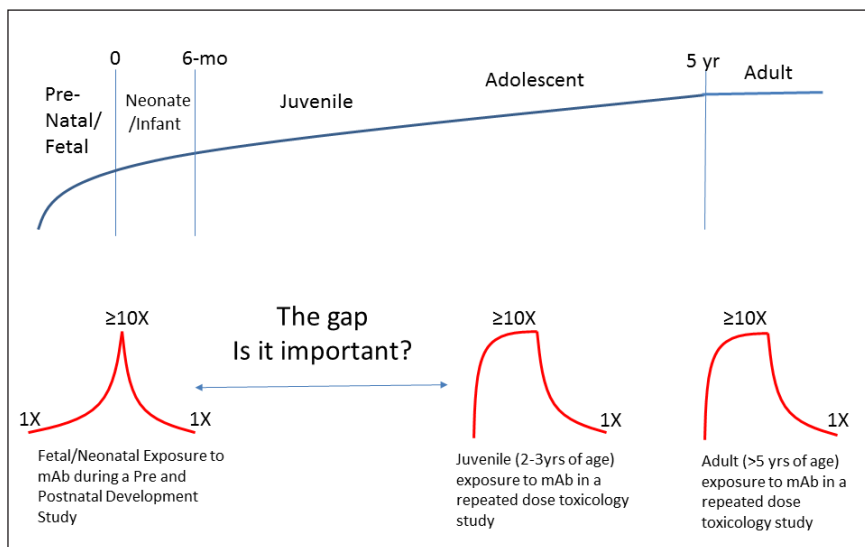
Figure 1:
Pictorial representation of serum concentrations of a mAb in the macaque fetus and neonate from an ePPND study and in the juvenile/adolescent and adult in a repeated dose toxicology study (lower panel. The relationship of these exposures relative to the developmental stages is depicted in the upper panel with the slope of the curve indicating a decrease in growth rate with advancing age.

An embryo/fetal developmental (EFD) study in which pregnant monkeys are dosed only during the embryonic period or during the embryonic and fetal periods with termination of fetuses at some time before birth may also provide some useful information for evaluating the potential susceptibility of the developing organ systems. Although exposure during the embryonic period (Day 20 – 50 of gestation in the macaque) is expected to be minimal for a mAb, the persistence of the mAb in the dam's serum during the fetal period will likely result in fetal exposure. However, macaque embryonic developmental studies in which the dams are dosed with a mAb only during the embryonic period and the fetuses are harvested prior to birth are rarely conducted anymore because continuation of dosing into the fetal period and postnatal examination provides more information regarding potential risks to fetal and neonatal development.

The fetal period in humans and NHPs (Gestation Day 50 through birth in macaques) is a period of extensive growth and maturation of the organ systems

that were formed during the embryonic period. For some of the organ systems this maturation process continues during the postnatal period but at a progressively decreasing rate. Macaques develop at a similar rate to humans during the embryonic period but at an accelerated rate thereafter. Consequently, at the time of birth macaques are either as developed or more developed than humans for most organ systems. As a consequence some of the events that occur postnatally in humans have already occurred prenatally in macaques and have therefore already been evaluated in the NHP developmental toxicity study. Also for those organs that continue to develop post-birth the most rapid maturation period is during the first 3–6 months in macaques and this corresponds developmentally to the first 2–8 years in humans.

5. Exposure to mAb during repeated dose general toxicity studies

In addition to the developmental studies, the general toxicity studies conducted in young adult animals may provide information that is relevant to the pediatric patient population. Macaques used for general toxicity testing are frequently within the 2–3 years age range. For females this represents a peri-pubertal age and for males it is a pre-pubertal age. Studies in these juvenile (adolescent) animals may be able to detect effects in the growing animal that cannot be detected in fully mature animals, for example effects on growth of the long bones or sexual maturation. If a combination of immature and mature macaques are utilized in the general toxicity studies then any potential age related changes in organ sensitivity can be evaluated.

6. 'The Gap' is it important?

The ePPND study with a mAb results in increasing exposure during fetal development and decreasing exposure for up to 6-months post birth. A 6-month general toxicity study will produce sustained exposure during the 6-month dosing period (unless intermittent dosing is the intended clinical regimen) followed by decreasing exposure during the treatment free recovery period. If the NHPs used in the general toxicity studies were 2–3 years of age at dosing initiation then there is a period of juvenile development between 6-months of age and 2–3 years of age when effects on development have not been examined (Figure 1). However, before embarking on a juvenile toxicity study to 'fill the gap' it is important to evaluate whether such a study is critical to understand the potential hazards to the pediatric population.

Filling the entire gap between exposure in a PPND study through to full sexual maturation would require dosing of NHPs for about 4–5 years. The only study that could rationally be done would be one in which a small window of exposure during a period corresponding to a comparable developmental age as the intended patient population was conducted. Based upon the organ systems discussion below this type of study may not to reveal new toxicities that had not already been identified in the ePPND and general toxicity studies. A juvenile toxicity in monkeys with a mAb might be most valuable for evaluating differences in the pharmacokinetics and pharmacodynamics in immature animals relative to mature animals that may necessitate dose adjustments in the clinic.

7. Postnatal development of the organs systems in humans and macaques

7.1 Organ systems that are well developed in the macaque at the time of birth or in within 6-months post birth

7.1.1 Respiratory tract

At birth the macaque lung is more developmentally advanced than the human lung (Zoetis and Hurtt 2003). In humans the alveolar stage of lung development is not complete at birth but continues until about 8 years of age (Thurlbeck 1975). In contrast the number of respiratory bronchioli and alveolar ducts in macaques is similar at birth and in the adult (Hislop et al 1984) and most of the differentiation of the tracheal epithelium occurs prenatally (Plopper et al 1986a,b). After birth the macaque lung mostly develops by growth of existing structures and increases in size with increase in body size. Despite this growth, there is no significant change in the average epithelial thickness (Van Winkle et al 2004). Some of the events that occur postnatally in macaques include mucus cell and gland development (Plopper 1986b), development of the lamina reticularis of basal membrane zone of the trachea (Evans et al 2002, 2010) and expression of Clara cell secretory protein, a protective protein secreted from nonciliated bronchiolar epithelial cells (Coppens et al 2009). These events mostly occur within the first 3-months post birth.

Because of the advanced stage of development of the macaque lung relative to the human lung, it has been suggested that the macaque is not an appropriate species for juvenile toxicity testing of products in which the lung may be a potential target organ for toxicity (Zoetis and Hurtt 2003). This is particularly the case for agents that are intended to be delivered by the inhaled route of admin-

istration. However, if the agent is intended to be administered systemically and a pre and postnatal development study has been conducted in which macaques were exposed during the fetal and early postnatal period, then this would likely cover many of the critical events of respiratory tract development that occur postnatally in human pediatric patients and no additional testing in juvenile animals may be necessary.

Overall, exposure of monkeys during fetal and early postnatal period (first 3-months) development covers many of the lung growth and maturation events that occur in pediatric patients up to 8-years of age (Figure 2) and therefore a juvenile toxicity to evaluate potential effects on the juvenile lung may be of limited value.

Figure 2:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neonate and adolescent relative to lung development.

7.1.2 Heart

Pre and postnatal development of the heart appears to be similar in humans and macaques (Hew and Keller 2003). In both species the heart is well developed at birth. Postnatal development is part of a continuum of changes that begins in the embryonic stages. In general, the period is characterized by the progressive addition and orientation of cellular components.

Overall, exposure of monkeys during the fetal period covers most of the major events in heart development relevant to pediatric patients. (Figure 3).

Figure 3:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neo-nate and adolescent relative to heart and kidney development.

7.1.3 Kidney

The pre and postnatal development of the macaque kidney is similar to that of humans (Zoetis and Hurtt 2003; Batchelder et al 2009, 2010, 2013; Tarantal et al 2001). In both species the kidneys are well developed at the time of birth. Nephrogenesis occurs during the fetal period and is complete prior to birth in both macaques and humans (Batchelder et al 2009, 2010, 2013; Tarantal et al

2001). During macaque fetal development the number of glomeruli per gram of tissue triples between gestation day 50 through 100 but remains relatively constant from gestation day 100 through term and through adult (Batchelder et al 2013). Immunohistochemical assessment of expression patterns of several proteins critical to the water balance function of the macaque kidney have been observed as early as the late first trimester, similar to that of humans (Batchelder et al 2010).

Overall, exposure of monkeys during the fetal period covers most of the major events in kidney development relevant to pediatric patients (Figure 3) and therefore a juvenile toxicity study to evaluate effects on kidney development may be of limited value.

7.1.4   Gastrointestinal tract

Postnatal development of the stomach involves thickening of the glandular region and the maturation of the pepsin producing chief cells (Walthall et al 2005). It is generally considered that in humans at birth the stomach of the newborn has low levels of acid and pepsin and the levels increase with age. In humans the levels of acid and pepsin reach adult levels between 3-months (Hyman et al 1983; DiPalma et al 1991) and 2 years of age (Deren 1971). In macaques (Macaca fuscata) the pepsin levels in the gastric mucosa of the stomach have been shown to increase from birth through 3 years of age (Kageyama et al 1991). The macaque studies further showed that the fetus-specific pepsinogens that facilitate the absorption of milk derived immunoglobulins in lower order species are not present in the monkey (Kageyama et al 1991). This supports the assertion that antibodies secreted in the milk are not absorbed across the primate neonatal gut.

The human small intestine is well developed at the time of birth (de Zwart et al 2004). The intestines grow in length and diameter during the postnatal period and mature functionally. By late infancy, most gastrointestinal functions are comparable to that of adults and in general species with long gestational duration exhibit postnatal development patterns more similar to humans (Waltham et al 2005). The permeability to macromolecules may be greater during the first 3-months post-birth than in adults (de Zwart et al 2004). This may be a consideration for therapeutic agents that are intended to be administered by the oral route. However, most protein therapeutics cannot be administered orally because they are too large to be absorbed across the gut and are degraded by pepsin to small peptides and amino acids that are small enough to be absorbed.

Overall, the gastrointestinal system appears to be well developed at birth or soon after birth in both macaque and humans. Therefore, for systemically administered agents, sufficient coverage may already have been obtained during the course of PPND studies and additional juvenile toxicity may not be needed (Figure 4).
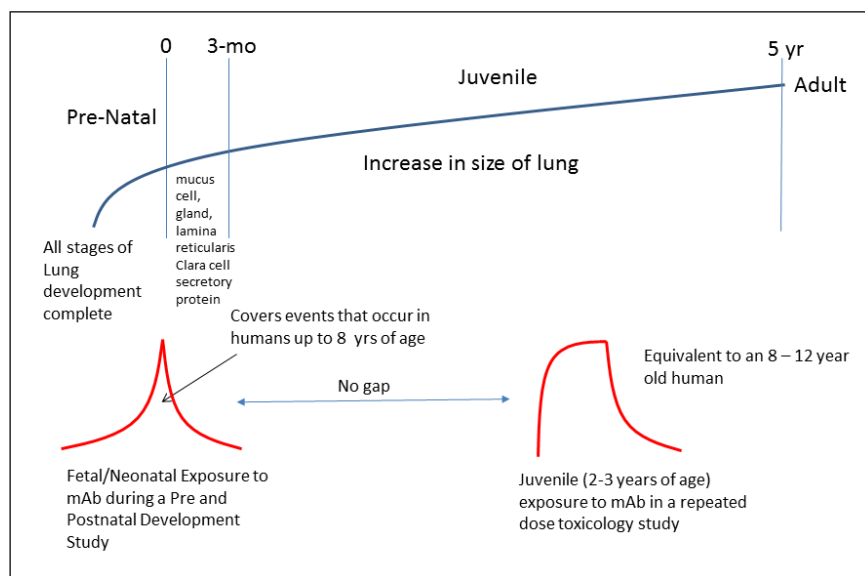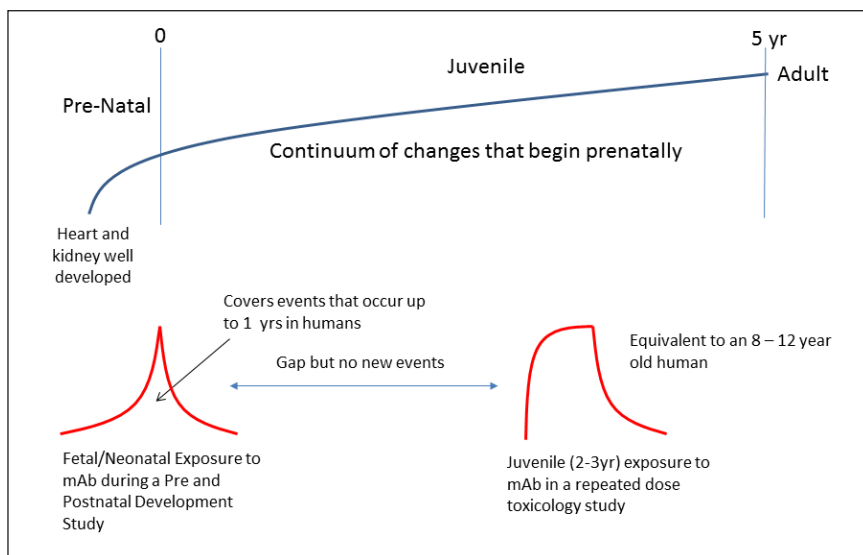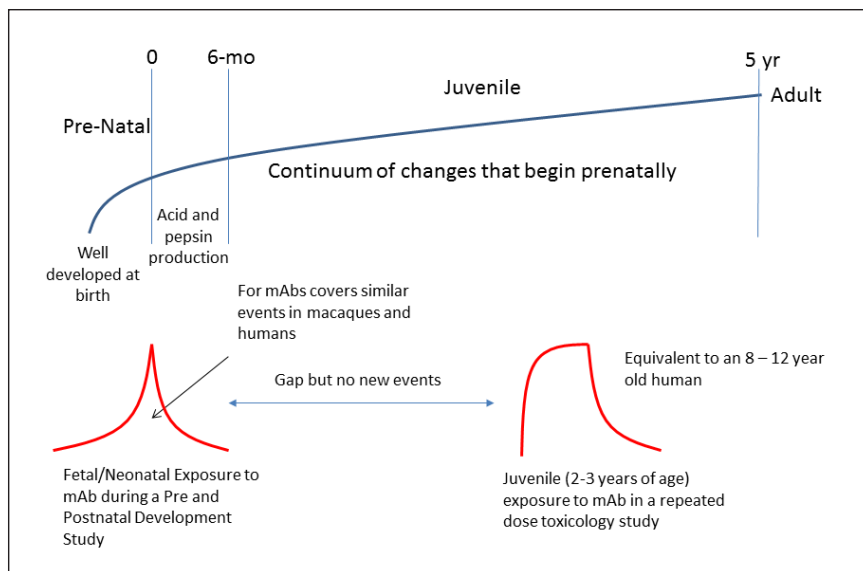

Figure 4:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neonate and adolescent relative to gastrointestinal development.

7.2 Organ systems that show significant development beyond 6-months of age

Although the organs described above are well developed at the time of birth or within the first 3-6 months post-birth there are some organs systems that continue to show sustained postnatal development through to adult. These include the reproductive system, certain parts of the central nervous system, the skeletal system and the immune system.

7.2.1 Sexual maturity

Many of the NHP studies exploring reproductive physiology have used the rhesus macaque. However, in more recent years the cynomolgus macaque has been more frequently utilized because this is the species of choice for the developmental and reproductive toxicity testing for biopharmaceuticals that have primate restricted pharmacology (Weinbauer et al 2013; Fuchs et al 2013).

Maturation of the zona reticularis of the adrenal cortex is essentially complete by 3-months of age in the rhesus macaque (Meusy-Dessole and Dang 1985). The increase in the secretion of adrenal androgens marks the maturation of the adrenal cortex (Conley et al 2011). In rhesus macaques these androgens peak at about 6 to 8 weeks of age in both males and females (Dierschke at al 1974; Terasawa and Fernandez 2001) whereas in humans the levels increase throughout the first 2 decades.

7.2.1.1 Male

A cross species comparison of the postnatal development of the male reproductive tract has been reviewed by Marty et al. (2003). In humans the testes become descended prior to birth. However, in macaques although the testes are descended at birth they regress postnatally and descend again at approximately 3 years of age (Wislocki 1933; Catchpole and Van Wagnen 1975).

The immature monkey testis is characterized by the presence of only Sertoli cells and undifferentiated spermatogonia (Dreef et al 2007). Up to the prepubertal period there is a slow increase in testicular weight, tubular diameter and number of seminiferous cords (Simorangkir et al 2012). The gonatotropin control of puberty in male rhesus macaques consists of two major postnatal phases of secretion. The first occurs during the first 6-months of age, peaking at about 3-months and the second begins at about 3-years of age, increasing through to adult. The second surge marks the beginning of puberty (Mann and Plant 2010). The gonadotropin surges are associated with high rates of Sertoli cell proliferation (Marshall and Plant 1996; Sharpe et al 2003; Plant et al 2005; Simorangkir 2003) whereas in the period between these surges, Sertoli cell proliferation occurs but at a reduced rate (Marshall and Plant 1996; Simoramgkir et al 2003, 2012). The proliferation of undifferentiated spermatogonia, is less dependent on gonadotropin stimulation than that of Sertoli cells. Spermatogonia in the testis become more numerous by the end of the first year but the earliest appearance of spermatozoa is approximately 3 years of age.

The steroid hormone producing cells of the testes, the Leydig cells, are prominent during fetal life in macaques. During the first year, the Leydig cells decrease in number and dedifferentiate entering a period of suspended development. By the end of the third year, the Leydig cells redifferentiate and begin to produce the primary steroid hormone testosterone (Catchpole and Van Wagenen 1975). The testosterone levels rise about 6-fold during sexual maturity in macaques (Bennet et al 1973).

A number of methods have been employed to determine the timing of sexual maturation in the cynomolgus macaque including age, weight (Smedley et al 2001), histopathology (Haruyama et al 2012a,b), orchidometry (Ku et al 2010), and presence of sperm in the ejaculate (Luetjens and Weinbauer 2012). Overall, the onset of sexual maturity in male cynomolgus macaques is approximately 4 – 5.5 years.

Overall, for males the period between 3-months of age and 3-years of age is a relatively quiescent with regards to testicular development in NHPs (Plant 2010). Changes in the seminiferous tubule are minimal and are limited to proliferation of sertoli cells and undifferentiated spermatogonia. Therefore, the most sensitive periods for potential disruption of normal testicular development would be during the first 3-month of life and during puberty (Figure 5).
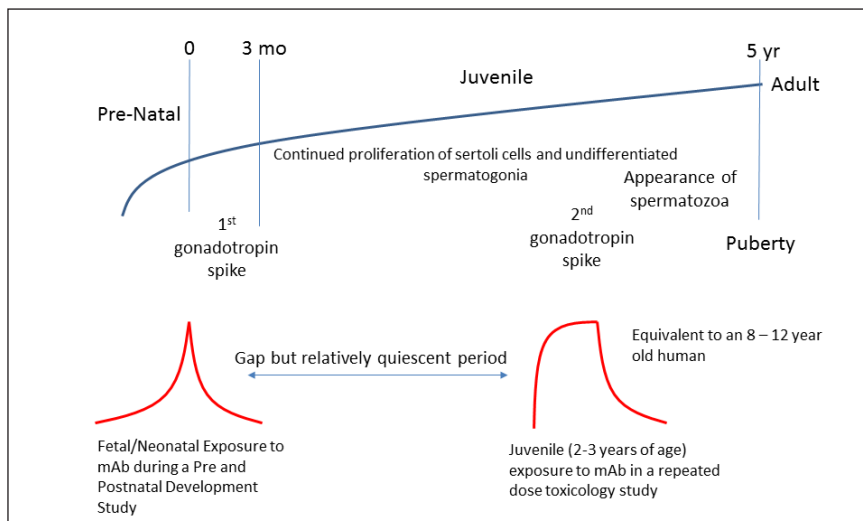

Figure 5:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neonate and adolescent relative to male reproductive tract development.

The first 3-months of life may be covered in the pre and postnatal development study whereas a period during sexual maturation may be covered in the general toxicity study in peri-pubertal monkeys.

7.2.1.2 Female

A cross species comparison of the postnatal development of the female reproductive tract has been reviewed by Beckman and Feuston (2003). In rhesus macaques menarche occurs at approximately 2.5 years of age Resko et al 1982). Following menarche there is a period of short luteal phases and anovulation before the monkeys develop normal menstrual cycles (Foster 1977; Rosenfield 2013). Because of this there is a lapse of about 12-18 months between the time of menarche and fertility (Resko et al 1982; Foster 1977). A postmenarcheal period of anovulation may also occur in humans (Rosenfield 2013).

Studies in the rhesus monkey (Terasawa and Fernadez 2001; Teresawa et al 1983; Kasuya et al 1999; Plant 2001) suggest that γ-aminobutyric acid (GABA) inhibits the gonadotropin-releasing hormone (GnRH) pulse during childhood. The onset of puberty is associated with a fall in GABA and increase in excitatory amino acids and the reactivation of the GnRH pulse generator (Ma and Ojeda 1997; Grumbach 2002). Estradiol concentrations increase in the initial stages of puberty at 2.5 to 3 years in NHPs (Terasawa et al 1983; Wilson et al 1986) and at 8 to 10 years in the human (Sizonenko 1978). In recent years it has been shown that kisspeptin is a critical regulator in the onset of puberty. Hypothalamic kisspeptin directly activates GnRH neurons to stimulate the reproductive axis and this occurs independent of the pubertal increase in circulating estradiol (Guerriero et al 2012a,b). Leptin may also be required to achieve puberty and to maintain cyclicity and reproductive function in NHPs and human (Chan and Mantzoros 2001).

Although puberty in both males and females is a postnatal event that could be affected by perturbations during childhood, the evaluation of sexual maturation and puberty in macaques is not feasible. Sexual maturation cannot be evaluated in pre and postnatal development studies in macaques because the time between birth and sexual maturity is about 3 years for females and 5 years for males. Clearly it is not practical to dose NHPs for this prolonged period of time. Also the exact timing of sexual maturation varies between animals so it would be difficult to design a study to capture the transition from immaturity to fecundity. In addition, reproductive potential cannot be accurately evaluated in NHPs because of their low natural fertility rate and high spontaneous abortion

rate (Chellman et al 2009; Martin et al 2009). For molecules in which there is a particular clinical concern based upon the known pharmacology hormones can be evaluated in immature and mature monkeys and this, in addition to histopathological examination of the reproductive organs, may give an indication of potential adverse effects on sexual maturation.

7.3   Brain

The postnatal morphological development of the monkey and human brain has been extensively reviewed by Watson et al (2006), and the functional development has been reviewed by Wood et al (2003). The monkey brain develops in a similar pattern to the human brain but there are some differences in the relative timing of the individual events. In macaques the most rapid growth in both total brain volume and white matter is from birth to approximately 4 months and is consistent with the emergence of cognitive abilities (Malkova et al 2006). In both humans and macaques neurogenesis begins early during embryonic development and continues during the fetal period (Difiglia et al 1980). At birth the majority of neurogenesis is complete with the exception of a few structures including the cerebellar cortex and the dendate gyrus of the hippocampus in which neurogenesis continues for some time post birth.

In both humans and monkeys there are considerable postnatal changes in hippocampal structure and function (Paule et al 2012). At birth humans have 70 – 80% of the adult number of dendate gyrus cells and the rhesus macaque has 50-60% (Coe et al 2003). In macaques the majority of the postnatal neurogenesis in the dendate gyrus occurs within the first 3-months but gradual increases continue to occur through about 1 year of age Jabes 2010). A similar pattern occurs in humans but extending through at least the first 5 postnatal years (Seress and Ribak 1995; Seress et al 2001; Seress 2007). A small amount of neurogenesis in the dendate gyrus may also continue throughout life (Watson et al 2006). Cell production in the cerebellum is completed after 2-3 postnatal months in the macaque but extends to the first postnatal year in the human infant (Warson et al 2006; Rakic et al 1994).

Synaptogenesis begins prenatally, increases through to infants (2 months in macaque, 2 years in humans) and then decreases such that adults have approximately 50% of the synapses that were present in infants (Rakic et al 1994; Levitt 2003). The process of reduction and refining of synapses from infant through to adult is referred to as pruning.

The functional abilities of the central nervous system develop postnatally in all species (Wood et al 2003). Non-human primates are more advanced at birth, or develop more rapidly, for many functional central nervous system endpoints relative to humans. In monkeys, the tactile, auditory and visual systems are more advanced at birth than in humans and locomotion and dexterity develop faster in macaques than in humans. Cognitive development occurs postnatally but cannot be directly compared with humans.

Although the developing brain may be more susceptible to perturbations than the adult brain, an important consideration is whether the molecule is able to gain access to the brain and then to interfere with cellular processes. The very large size of mAbs (~150 kDa) generally precludes them from traversing the intact blood brain barrier to any great extent (Partridge, 2012). There have been some conflicting reports regarding when the blood brain barrier becomes fully developed. Studies conducted in non-primates have suggested either that the blood brain barrier is fully developed at birth or incompletely developed at birth depending on species and methodology (Grotoft 1954; Adinolfi 1985; Mollgard and Saunders 1986; Rodier 1995; Costa et al 2004; Watson et al 2006; Ek et al 2012). No information could be found for NHPs and the few
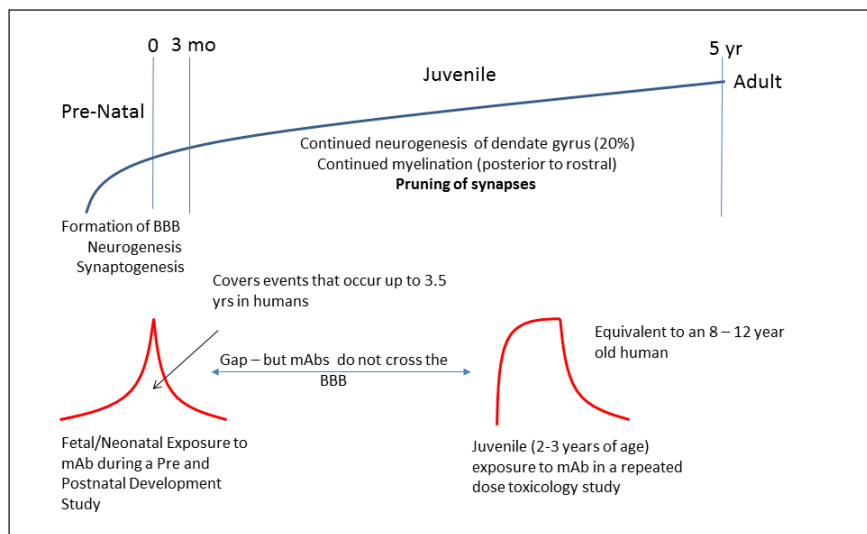

Figure 6:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neonate and adolescent relative to brain development.

studies conducted in humans have suggested a fully developed blood brain barrier at birth (Ek, 2012).

Overall, although a considerable amount of development of the central nervous system occurs postnatally in macaques (Figure 7) and humans it is unlikely that a mAb will have an effect on brain development in human pediatric patients because the blood brain barrier will prevent access of the mAb to the brain tissues. An exception would be for a mAb that had been specifically engineered to cross the blood brain barrier (Pardridge, 2012). However, even if a small amount of mAb is able to access the brain a NHP study to evaluate effect on cognitive function may be of limited value because the tests available for learning and memory in monkeys are relatively insensitive (Cappon et al 2012).

7.4   Bone

Bone development is generally more advanced in monkeys than in humans at birth (Zoetis et al 2003). The bones begin to form during the embryonic period with the primary ossification centers appearing during the embryonic and early fetal period. In macaques secondary ossification centers are either present at birth or appear within the first 6-months postnatally. Bone ossification in the macaque at birth is equivalent to that of a 5- to 6-year-old human (Michejda 1980). During the postnatal period, long bones increase in length at the epiphyseal growth plate. The bones continue to increase in length until the growth plate becomes fused in adulthood. Increases in bone diameter occur by deposition of new bone on the periosteal surface. The cessation of long bone growth with epiphyseal fusion has been reported to occur between about 2 to 6 years in macaques (Zoetis et al 2003; Fukuda et al 1978; Fukuda and Matsuoka 1980). Lengthening of the long bones occurs at a relatively steady rate throughout the postnatal period in macaques with the exception of an initial growth spurt during the first year and a second spurt during puberty (Watts and Gavan 1982). Postnatal growth and development of the macaque jaw bone occurs to the greatest extent during the first year (McNamara and Graber 1975).

Skeletal growth and maturation is a continuous process that occurs during the pre and postnatal period through to adult. Therefore, disruptions in this process at any time during development could potentially affect growth. However, disruptions in the prenatal and early postnatal periods are likely to have the most notable effects since this is when the skeletal structures are being formed and the ossification centers appear. Therefore, if a PPND study is conducted in macaques and fetal and neonatal exposure occurs without adversely affecting

Figure 7:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neonate and adolescent relative to skeletal development.

skeletal development then it is unlikely that exposure later in development will reveal new toxicities. Any later toxicity is likely to be similar to those observed at earlier time points but to a lesser extent. A good example of this is the class of angiogenesis inhibitors that produce marked skeletal abnormalities when exposure occurs during the prenatal period due to a disruption of the blood supply to the developing bones. However, when exposure occurs in juvenile animals an interruption in growth of the long bones can occur by a similar mechanism but this effect is less dramatic than that observed in the fetuses and may be reversible upon cessation of treatment (Ryan et al 1999; Patyna et al 2008).

Overall, exposure of monkeys during the fetal period covers a sensitive period in bone maturation that is equivalent to events that occur in pediatrics up to 6-years of age (Figure 8). Therefore if skeletal effects have been observed in an EFD or PPND study with or without effects in a general toxicity study in adolescent animal then the hazard will already have been identified and additional studies in macaques between 6-months and 2 years of age may not add significant value.

7.5 Immune system

The prenatal development of the macaque immune system is very similar to the human immune system (Makori et al 2003; Buse 2005). At birth both macaques and humans have a well-developed immune system with regards to the cellular components and have the potential to generate immune responses (Holsapple et al 2003). All of the major cell types, T, B and natural killer cell (NK) cells, are present before birth and demarcation of the splenic architecture occurs during the fetal period.

Immune competence becomes established postnatally. Postnatal development of the immune system has been studied using various approaches such as blood immunophenotyping, T cell-dependent antibody response (TDAR), analysis of lymphocyte proliferation and NK activity, and immunocytochemical localization of immune cell populations (Buse 2005; Henrickx et al 2002; Burns-Naas et al 2008; Weinbauer et al 2008). These data collectively demonstrate general functionality of the cynomolgus monkey immune system in early postnatal life and striking developmental similarities to human immune system development.

Serum immunoglobulin (Ig) levels can be used as a marker for age-related humoral immune function in the cynomolgus monkeys (Buse 2005; Terao 2009). At birth serum IgG levels are high and serum IgA and IgM levels are low. The IgG in the serum at birth is most likely to be maternally derived since IgG, but not IgM or IgA, can cross the primate placenta during gestation. The IgG concentrations decrease during the first 6-months of life due to clearance of the maternal antibodies from the infant's serum and then rise thereafter when the monkeys start to generate their own IgG (Buse 2005; Terao 2009). IgA and IgM levels increase from birth through to adult. The immunoglobulin concentrations in the serum reach the adult level by about 5–9 years of age suggesting that the immune system is continuing to mature during this time period (Terao 2009). Analysis of lymphocytes for cell surface markers have shown that the relative proportion of lymphocyte subsets with resting phenotype decreased with increasing age, while the subpopulations associated with activation gradually increased with age reaching adult levels by about 5–10 years of age (Terao 2009). Although the immune system matures from birth through to adult, the first 3–6-months is the most critical period in the acquisition of immune competence (Zhu et al 1997, 2000; Polack et al 2013; Pan et al 2005; Lockridge et al 1999; Martin and Buse 2008).

Overall, the prenatal development of the immune system is similar in macaques and humans and is well developed at birth. Immune competence becomes es-
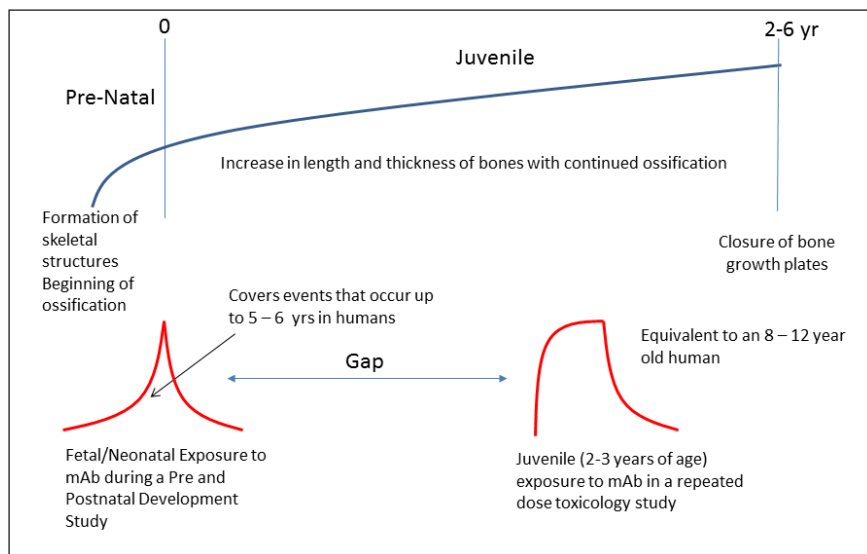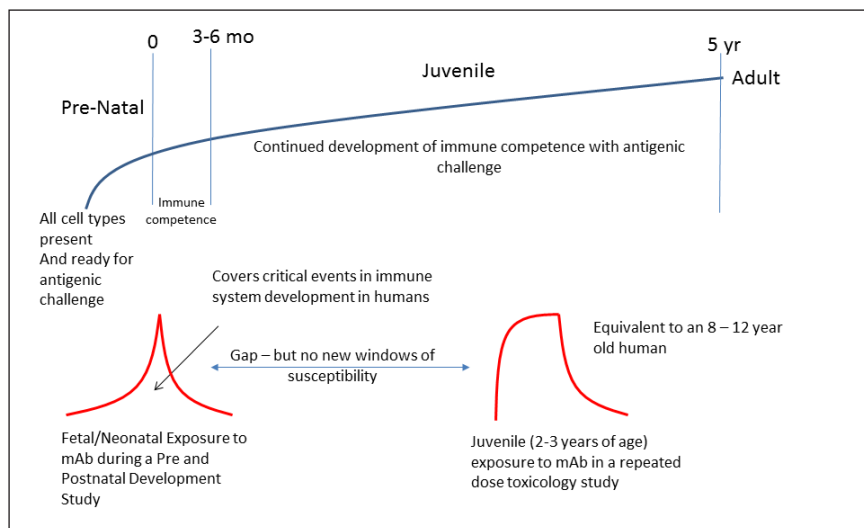
Figure 8:
Pictorial representation of serum concentrations of a mAb in the macaque fetus, neonate and adolescent relative to immune system development.

tablished during the first 3-6-months after birth. Therefore, exposure during the fetal period and the neonatal period in macaques covers sensitive periods in immune system development that are relevant to the pediatric population (Figure 8) and additional studies in juvenile animals may not be necessary to evaluate potential effects on the developing immune system.

8. Rationale for not conducting a non-clinical study in NHPs – the weight of evidence approach

For most organ systems, the events that occur postnatally are part of a continuum of events that were initiated during the embryonic stage of development. The growth and maturation of the organ systems occurs at the greatest rate during the fetal period and the early postnatal period. For macaques the most rapid period of postnatal development occurs during the first 3-months, with the exception of the reproductive system that matures at puberty. The period from 3-months to 3-years in macaques represents a protracted period of development in which a slow steady progression in the maturation of the organ systems occurs. Therefore, for those organ systems that are relatively well developed at

birth or soon after birth and have been evaluated for effects on embryo/fetal development in a PPND study, the conduct of additional juvenile toxicity studies may not provide sufficient added information to justify the conduct of the study.

A review of all of the available non-clinical and clinical information provides the basis for the weight of evidence approach for supporting pediatric clinical indications. The supporting data would include a review of the available pharmacology of the molecule and similar molecules in its class, a review of any available clinical information and a review of the toxicology data from general and developmental toxicity studies. This review may provide sufficient information to understand potential risks to the pediatric patient such that additional studies in monkeys are not necessary.

An important consideration, however, is the timing of the monkey developmental studies relative to pediatric clinical development. The ICHS6(R1) regulatory guidance document states that the developmental toxicity study can be conducted during Phase III. This would allow Phase II clinical proof-of-concept studies to be completed before the large and costly monkey developmental toxicity study is initiated. For those products that have both adult and juvenile indications, demonstration of clinical efficacy in adult patients often precedes studies in pediatric patients and therefore the developmental studies will be ongoing or completed before pediatric patients are recruited into clinical trials. Also it is common for pediatric clinical development to recruit adolescent patients first and then to 'step down' to younger patients as acceptable patient safety is demonstrated.

9. Rationale for conducting a non-clinical study in NHPs

When the therapeutic agent is intended to be administered only to pediatric patients, then a justification could be made for conducting all of the general toxicology studies in juvenile animals at a developmentally equivalent age. Depending on the age of the target patient population, a developmental toxicity study may not be required, although as described above it could provide valuable information for the pediatric patients especially if the potential target organs develop postnatally in humans but prenatally in monkeys and the NHP is the only pharmacologically relevant species.

10. Conclusion

An assessment of potential risk to postnatal and juvenile development needs to be considered prior to the inclusion of children in clinical studies. If the existing nonclinical data is insufficient to justify the use in the intended patient population, then a juvenile toxicity study may be warranted. The non-human primate is not the recommended species for juvenile toxicity testing, but may be used when scientifically justified.

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Considerations in the Design of Molecule-specific Preclinical Testing Strategies in NHP to Enable Pediatric Clinical Trials

Wendy G. Halpern

Abstract

The thoughtful design of a general nonclinical testing strategy should include consideration of potential pediatric use. Current regulatory guidance and law specifically requires agreement with US and EU Health Authorities regarding the pediatric strategy, including discussion of completed and planned non-clinical studies to support pediatric use. This chapter will highlight some challenges of that come with juvenile toxicity testing, especially when NHP are the only pharmacologically relevant test system. Interpretation of results requires an understanding of translatability of drug effects on developing organ systems, and can be complicated by species differences. If juvenile studies are conducted, it is critical to link the results with the available nonclinical safety profile from completed general toxicity studies, and with the clinical safety profile. There are also instances where additional studies in juvenile animals are unlikely to provide additional information, in which case a clear scientific rationale for not including a juvenile toxicity study will be expected. In general, juvenile toxicity studies should be conducted for cause to identify drug effects that may be specific to a pediatric patient population. The ultimate goal of these studies is to influence the design of an appropriate clinical pediatric testing program, and label designation for informed pediatric use.

1. Introduction

In current practice, most drugs being developed initially focus on diseases in adults. However, there may be relevant pediatric diseases as well. In some instances, such as juvenile rheumatoid arthritis or asthma, the pediatric disease may have substantial overlap with the adult indication. In other instances, such as oncology, there may be very little overlap for specific indications. The timing and approach to support of clinical studies in pediatrics will vary considerably

based across programs, but one consistent feature is that every program needs a pediatric strategy – even if the strategy is to strongly recommend against pediatric use for some therapeutics.

The pediatric strategy often includes consideration of the conduct of a juvenile toxicity study. It is important to ask first whether such a study is warranted, and how the additional data will add value to the pediatric risk assessment. There is no single 'correct' design for a juvenile toxicity study, especially in NHP, which mature over several years. The context of the program, including the expected pharmacology, potential impact on development, and developmental ages and stages of the clinical population, should all be considered in the design to ensure availability of the most useful data.

In developing a pediatric strategy, many project teams have concerns around regulatory risk. It is important to initiate discussions with Health Authorities with adequate time to adjust plans if necessary. Nonclinical activities supporting the development of a pediatric plan may need to be planned or initiated relatively early in the product development process. This is particularly true for immediately life threatening diseases for which no efficacious therapy exists. In this case, there may be a strong case for earlier access to promising new medicines.

2. Challenges in nonclinical support of pediatrics

In considering approaches to pediatric drug development, the global regulatory guidance for nonclinical safety assessment has been somewhat mixed, and there is currently no harmonized nonclinical guidance for nonclinical support of pediatrics. The ICH M3(R2) states that *'nonclinical juvenile toxicity and/ or DART studies should be designed to identify juvenile-specific effects, not confirm'*. This is consistent with the recommendation to avoid the conduct of juvenile animal studies when adequate clinical or nonclinical data already exist for risk assessment. It is also consistent with a case by case approach in considering the value of conducting a juvenile animal study. However, there is room for breadth of interpretation in terms of a requirement to identify juvenile-specific effects, and the focus of juvenile toxicity testing is often centered around a rodent test system (Brent, 2004; Robinson, 2008; Baldrick, 2010; Barrow et al, 2010).

Most publications acknowledge the limitations of the available test systems despite the desire to provide comprehensive support for pediatric risk assess-

ment. In many cases, there is not a strong scientific argument for the conduct of juvenile animal studies, yet such studies may still be expected or required. This uncertainty leads to anxiety around regulatory risk, which then influences the nonclinical strategy. In addition, initial consideration of the pediatric strategy often coincides with other critical development activities exploring efficacy in adults. Unfortunately, these factors tend to result in a defensive and reactive, or highly conservative, approach to pediatric support. Before initiating a study in juvenile monkeys, there should be a balanced consideration of the scientific merits and design to best inform pediatric use.

We know that there is no nonclinical test system that can fully model pediatric risks, but the nonhuman primate is considered relatively close for many developmental milestones. However, working with the juvenile monkey test system is also challenging. As with most other nonclinical safety studies in NHP, group sizes are small, and it may be difficult to achieve rigorously matched age and gender balance across groups. These studies are most useful in hazard identification rather than comprehensive risk assessment. Unlike rodents, which mature completely a few months after birth, it is not practical to evaluate the full 0-6 year 'pediatric window' in macaques to cover birth through 18 years of age in humans. In addition, due to weaning time (typically 3-6 months after birth), shipping regulations, and quarantine/acclimation time, it is most practical to conduct juvenile monkey studies with animals that are 9-12 months of age at study start. Thus, it is important to be aware of the developmental processes still going on at that time in macaques, and relevant new information that may be obtained.

In considering a study in juvenile monkeys, there are a few 'bottom line' questions:
• What can we learn that has not already been evaluated in other studies?
• How translatable are the findings we generate?
• How will pediatric use of the specific test article be informed?

The first step is thoroughly investigating what is already known about the molecule, the targeted pathway(s) and the relevant pediatric population. Prior to initiating human studies, there has often been considerable nonclinical testing to characterize the mechanism of action (MoA) for novel candidate therapeutics. The MoA needs to be considered both in selecting appropriate patients, and in predicting risk of unwanted toxicities. When the MoA suggests the potential for different effects in pediatrics than adults, this should be part of the risk assessment. There may also be information available about the expression of the target or activity of the targeted pathway in pediatrics versus adults. If

not already available, in vitro screening of normal and or diseased tissues thought to express the targeted pathway may be considered. In addition to risk assessment, such data could help pick an appropriate group of pediatric patients, or contribute to selection of a diagnostic biomarker. Finally, understanding the MoA and target/target pathway expression may help in identification of potentially relevant pediatric disease models. This applies both to settings where the disease in children is similar to that in adults, as well as where it may be different. For example, in oncology, there are largely non-overlapping tumor types between pediatrics and adults. Understanding the pharmacologic relevance of the candidate therapeutic is critical in building a solid risk:benefit assessment.

The next step, drawing on the pharmacology, is to try to define the risks specific to pediatrics. For example, there are often genetic models in rodents that may provide some limited safety information. Understanding the translatability of these models is difficult – if there are severe effects, is it just because of a susceptibility window early in development? Likewise, if there are no discernible effects, is it because of an early compensatory response? Is the model relevant to primates at all? This will depend to a large extent on the molecule, the specificity of effects and the targeted pathway. However, using one or a combination of targeted knockout, knockdown or activating manipulations, in traditional or conditional settings, a picture of potential developmental liabilities may be established. Ultimately, these models are mechanism-based, not molecule-based, but can still contribute to the risk assessment of molecules or molecule classes.

A thorough understanding of the MoA and the potential developmental liabilities of a particular targeted pathway is the start to enabling appropriate pediatric use of a new therapy. In many instances, no additional nonclinical safety studies may be warranted or useful in further developing the clinical risk assessment, especially when a large animal test system, such as NHP, are required for relevant pharmacology. Both potential to unveil developmental effects important for pediatric use and translatability should be considered cornerstones of the decision to move forward with a juvenile monkey study.

3. Characterization of developmental toxicity: when are nonclinical studies most useful?

Comparative maturation by organ system is discussed in detail in another chapter of this publication (Martin P), and in a series of articles published between 2006-2011 in Birth Defects Research Part B (Beckman et al 2003,

Hew et al 2003, Holsapple et al 2003, Marty et al 2003, Wood et al 2003, Zoetis et al, 2003a, Zoetis et al 2003b, Zoetis et al 2003c, Walthall et al 2005, Watson et al 2006). There are two very general ways that maturation may influence the safety and pharmacology of a drug: 1) developmental effects on the absorption, distribution, metabolism and excretion (ADME) of a drug, determining the pharmacokinetics, and 2) effects on developmentally sensitive cell populations or tissues leading to novel toxicities not identified or predicted by effects on adult animals or patients.

3.1   Developmental effects on ADME

Since exposure is often a primary driver of toxicity, developmental effects on ADME must be considered for pediatric use. The maturation of the gastrointestinal tract may affect oral bioavailability, gut pH and flora; likewise metabolism and clearance may depend on hepatic enzyme function. The maturation of the CYP450 system in young monkeys has been investigated (Ise et al 2011), and results reflect both similarities and differences with what is known for human CYP450 maturation in terms of expression and activity in the early postnatal phase. The urinary and respiratory system may also contribute to drug clearance, potentially leading to higher or prolonged exposure when immature. In monkeys and humans, nephrogenesis is complete prior to parturition, but glomerular filtration rate and other functional endpoints continue to develop in the postpartum period (Zoetis et al 2003c). In general, pulmonary development is considered complete in monkeys at the time of parturition, but continues postpartum in humans; thus, monkeys are not considered ideal to assess inhaled formulations of drugs intended for use in neonates or infants (Zoetis et al 2003b). Maturation of the blood-brain barrier and organic anion transporters influences distribution, or lack thereof, to the central nervous system (Watson et al 2006). Other factors such as general membrane permeability and serum protein binding may also affect the amount and distribution of active drug. Finally, there are basic differences in body composition and basal metabolism between juvenile and adult animals and humans that should be considered in selecting appropriate doses for pediatrics (Xu et al, 2013). Importantly, depending on both the properties of the drug and the developmental stage of pediatric population, these developmental effects may result in either increased or decreased exposure relative to adults.

From a safety perspective, it is desirable to generate data that may help avoid acute toxicity due to higher than expected exposures in children. However, it is also important to be open to exploring higher dose levels in children than

adults if required to generate exposures in the predicted efficacious range. For some molecule types, such as monoclonal antibodies and other biotherapeutics, the expected PK for the molecule class are well characterized over a broad age range in humans, and large shifts in PK from predictions are unlikely and may not warrant additional nonclinical in vivo studies to study exposure (Morford et al 2011, Xu et al 2013). For small molecule therapeutics, an understanding of the ADME properties in adults can help highlight potential developmental exposure liabilities. In monkeys, developmental effects on ADME are expected to be different from adults predominantly in the pre-weaning phase when enzyme functions and tissue barriers are still actively maturing; in addition, juvenile monkeys tend to be more precocious than their human counterparts, with nonlinear developmental milestones across organ systems. In general, understanding ADME may be most important for the very young (neonates and infants). However, such studies may be difficult to conduct, as manipulation of mother/infant pairs of monkeys should be minimized in the early postpartum phase. While it is critical to be aware of the many factors contributing to exposure, there is still no single test system that can consistently model all potential developmental ADME effects to predict human exposures.

3.2   Developmentally sensitive targets

In considering the value of nonclinical studies in juvenile animals, one point often made is that there are potential targets in a growing animal that may not be relevant in the adult population. This general concern should be balanced by what is already known, or expected to be learned, from the conduct of such a study. Some organ systems which are considered of special interest during development are the musculoskeletal system, neurologic system, reproductive and endocrine systems, immune system, and cardiovascular system.

General growth and development are possibly most simply modeled by tracking effects on the *musculoskeletal system*. Unfortunately, the growth curve relative to gestational age/parturition and sexual maturity are not readily scaled to age or species buckets, and studies in nonhuman primates may not be adequately powered to detect small effects. However, when the musculoskeletal system is specifically targeted, the severity of the effects may depend on the relative maturity of the animal or patient. Like humans, nonhuman primates undergo endochondral ossification for elongation of long bones such as the humerus and femur, and have a similar distribution of woven, compact and cancellous bone during development. The neonatal macaque has a skeletal structure comparable to that of a 5-6 year old child, but complete skeletal maturation takes several

years, with epiphyseal closure occurring at 5-7 years of age in cynomolgus monkeys (Zoetis et al, 2003a). Thus, even nonclinical studies conducted in sexually mature monkeys may include animals with active growth plates. If documented, this can help in the assessment of potential effects on bone growth that may translate to growth disturbances in children. Likewise, assessment of effects on body weight and bodyweight gain in all nonclinical studies may be directly relevant for potential effects on growth in pediatrics.

Development of the *neurologic system* poses some challenges when evaluating data from nonhuman primates. It is well established that critical anatomic and functional changes occur postnatally in both humans and nonhuman primates (Wood et al 2003; Watson et al, 2006). These include both a marked postnatal expansion of synaptic connections early on, a plateau phase, and finally a reduction in connections. There are also gradual migration and pruning processes that are ongoing in the developing CNS. However, these changes are very gradual, taking place over relatively long periods of time, and it may not be possible to distinguish individual animal variability from subtle test article effects using standard study endpoints such as histopathology. Robust functional assessments of cognition have also been difficult to establish in nonhuman primates (Cappon et al, 2012).

Clear developmental changes occur around the time of puberty; thus the potential developmental effects on the *male and/or female reproductive system* may be of particular concern in supporting pediatric use. There are differences in the timing of expansion of specific cell populations, including both germ cells and stromal/ support cells. Although there are dramatic shifts in hormone production, spermatogenesis, and menstrual cycling during puberty, steroid hormone production and regulation is also important during childhood. In general, monkeys are a good model system for detection of reproductive effects that are directly relevant to humans. However, there are some exceptions. For example, adrenarche occurs in humans in middle childhood (5-7 years of age), but is not a feature of macaque maturation; in fact, steroid hormone production in juvenile macaques is comparable to that in adults beginning in the early postnatal period (Beckman et al, 2003; Marty et al, 2003).

Assessment of potential reproductive effects is complicated by the fact that many, even most, biopharmaceuticals have some *potential* to affect the reproductive system. Growth factors, growth factor inhibitors, cytokines, cytokine antagonists, immunomodulators, and drugs affecting metabolism may all have direct or indirect effects on reproduction. Although reproductive organs are routinely examined in general toxicity studies, the developmental stage

is not always captured. If effects are identified in reproductive tissues, it is important to know what developmental stage was evaluated. Inclusion of immature and mature NHP in general toxicity studies may adequately inform programs in terms of general reproductive risk, but these assessments are not as comprehensive nor as well powered as similar studies conducted in rodent test systems.

Immunomodulatory biopharmaceuticals are relatively common and often require an NHP test system. Differences in juvenile and adult *immune system* components, potentially affecting both anatomy and function, have been described (Holsapple et al, 2003; Morford et al 2011; Chellman et al 2009). Importantly, the general developmental stages are similar in macaques and humans. Although both are somewhat vulnerable in the neonatal period, neither is immunoincompetent. While the immune system continues to develop and mature through adolescence, the main differences reflect antigenic experience rather than a lack of specific cellular components. General effects on the immune system are therefore likely to translate across age ranges, so anticipating potential consequences in the pediatric setting is an important aspect of the risk assessment. For example, children may be more likely than adults to develop community acquired infections.

The *cardiovascular system* is of particular developmental interest in children, both in terms of potential acute and long term effects. The ECG is not considered mature until ~5 years of age in humans, and the heart mass continues to expand through adolescence. In monkeys, the heart mass grows as a function of general growth and development, but conductance is comparable to adults as early as 3 months postpartum. In fact, one of the pivotal studies evaluating normal ECGs in radiotelemetry instrumented macaques was conducted using immature (13-15 month old) macaques (Gauvin et al, 2006). Unfortunately, one would typically need to follow juveniles out through full maturation to understand potential long term effects on cardiac contractility and function – and in most cases this is not practical for studies in monkeys.

4. Example of nonclinical safety data in support of pediatric use of a monoclonal antibody for a chronic disease

For many monoclonal antibodies, the cynomolgus monkey may be the only pharmacologically relevant nonclinical species. For this example, it is assumed that both binding and activity are comparable between humans and cynomolgus monkeys. A general toxicology program to enable initial human clinical

studies would typically include a tissue cross reactivity study or comparable demonstration of potential binding across a broad variety of tissues, a single dose PK/PD study in monkeys and a repeat-dose toxicity study including a recovery phase. The human tissues available for tissue cross reactivity studies are donated surgical or cadaveric tissue specimens; these are typically from adults, and often from geriatric individuals. Pediatric-origin tissues may be requested, but are difficult to procure and are generally not available for routine studies. In contrast, if a parallel set of tissues from cynomolgus monkeys are available, they are often from young or young adult animals.

Unless otherwise prespecified, the monkeys used in the in vivo studies are often immature or peripubertal (2-4 years of age), and in many ways are comparable to school-age children (approximately 4-16 years old). The initial repeat dose toxicity study often includes a dosing phase of 4-12 weeks in duration, with a subset of monkeys allowed to undergo a treatment-free recovery phase prior to necropsy. The duration of the recovery phase is often driven by both the expected drug elimination kinetics and expected target tissue effects, but is usually also 4-12 weeks in duration.

As molecules advance through clinical development in adult patients, additional toxicology studies may be conducted to support administration of the drug for longer treatment duration and/or to a broader patient population. When the nonclinical safety studies are conducted exclusively in nonhuman primates, efforts often include an integrated approach for some study endpoints as a way to reduce animal use. For example, if sexually mature monkeys are prespecified for inclusion in a chronic repeat dose toxicity study, reproductive/fertility parameters may be included as well. If effects on the reproductive system are expected, a relatively long (4-6 month) recovery phase may be needed to assess the reversibility of effects on the reproductive system.

Additional studies may be appropriate, depending on the mechanism of action, adult indication and clinical safety profile. If the patient population includes reproductively competent men and/or women, developmental and reproductive toxicity studies may be needed. Especially when restricted to NHP, this may include a pre-and postnatal development study (enhanced design), or ePPND study (Weinbauer et al, 2011). For compounds that are well tolerated during pregnancy, the postnatal phase of this type of study may be useful in assessing developmental liabilities in the neonate or infant offspring. Effective interpretation requires that exposures of offspring are documented and/or predictable based on molecule class.

In looking for teratogenicity, radiographic skeletal assessments of offspring are usually conducted approximately 7d postpartum. If no skeletal teratogenic effects are identified, more general effects on growth and development may be monitored postpartum. This period may also include neurobehavioral, immunotoxicity, and cognitive (learning and memory) assessments. At the end of the 3 to 12 month postnatal observation phase, a full necropsy can be conducted to evaluate potential teratogenic effects on soft tissues. Although not routine for all sponsors, comprehensive gross and microscopic pathology of offspring may be conducted. If these data are intended to directly support pediatric use, it can be useful to evaluate both target and non-target tissues. Overall, assessment of offspring in an ePPND study where exposure is confirmed, in addition to inclusion of sexually and or skeletally immature monkeys in the general toxicity program, is an effective bridge to the identification of potential developmental liabilities.

Human organ system development follows several general stages (Robinson et al, 2008), with full maturation of the renal, pulmonary and gastrointestinal systems by 2 years of age; the cardiovascular and immune systems by 5-12 years of age; and continued development of cognition, general growth/skeleton, and the reproductive system through adulthood. Given the precociousness of monkeys at birth, there are fewer differences between juveniles and adults in the postweaning phase, although general growth, reproductive maturity, and expansion of the immune repertoire based on antigenic experience continues for several years. Given these species differences, and a non-immediately life-threatening disease context, investigative clinical pediatric studies may follow an age-gated step-down approach. For example, first children from 12-18 years of age, largely supported by the general toxicology program and accumulated clinical data from adults, would be studied. This population might then expand to include 6-11 year olds, and then, if warranted, 2-5 year olds or 0-5 year olds.

5. Additional challenges in oncology

In considering pediatric use of oncology drugs, several challenges arise that differentiate this setting from the previous drug development example. Oncology drugs, almost by definition, have potential for nonclinical and clinical toxicity. By targeting dividing and undifferentiated cells, such as tumor cells, it follows that unintended targets will include developmentally sensitive tissues. Thus, in considering pediatric use of oncology drugs, the starting point is typically one of known or expected risk. In addition, although there may be safety and efficacy data available from adult clinical trials, the specific tumor

types in pediatrics vs adults rarely overlap. If the tumor type studied in adults occurs in the lung, breast or colon, but the pediatric setting includes brain tumors and leukemia, it is likely that there will be differences not just in age but also in the disease presentation, progression and typical adverse effects. Furthermore, the efficacy profile may have a strong relationship with tumor type, and may not be directly comparable between adult and pediatric patients. Finally, the biomarker strategy to predict potential responders in the adult trials may not be relevant or useful for pediatrics. These differences between adult and pediatric tumors make it much more difficult to determine with confidence which pediatric oncology patients are most likely to benefit.

Establishing a risk:benefit assessment is further complicated because an unselected pediatric oncology population is likely to include a broad age range (potentially 0-18 years, although mean ages are usually older in the relapsed setting), may require a novel pediatric formulation, and is likely to include provision for repeated dosing in the absence of disease progression. Fortunately, the number of pediatric oncology patients is small, yet their need is great; single-dose clinical PK studies are not ethically a good option. Finally, the EU nonclinical guidance provides for 'safe use OR no effective therapy', while the FDA recommends nonclinical identification of toxicity specific to pediatric use.

From a nonclinical safety testing perspective, and acknowledging that drugs intended to treat indications that fall under ICH S9 are often toxic, it is important to set expectations regarding the utility and design of a juvenile monkey study:
Is there a path to 'safe' pediatric use, and is a juvenile toxicity study always required?
If not, what body of data is adequate and appropriate?
If toxicity is expected and somewhat generalized (e.g., cytotoxic drugs), how will additional nonclinical studies specifically inform the *pediatric risk assessment*?
How will nonclinical toxicity assessment inform *clinical use and monitoring* for pediatrics?

Additional considerations for pediatric use include strategy for dose selection (weight-based, age-based, BSA-based, adapted flat dose), route of administration and dose volume, and potential development of a pediatric formulation.

The thoughtful development of a pediatric strategy for oncology drugs needs to rely heavily on understanding the mechanism of action for each molecule

and relevance to specific tumor types. A robust nonclinical safety assessment in juvenile animals may not always be feasible or warranted, but the rationale for the nonclinical strategy should include consideration of the value of such studies.

6. Summary

The first and most critical step in considering pediatric drug use is the assessment of molecule-specific information already available:
• Species considerations for toxicity studies
• Completed studies (and age/developmental stage of animals)
• Summary of adverse effects (nonclinical and clinical)
• Any effects on developmentally sensitive targets?
• Known/expected pharmacology on developmentally sensitive targets?

Identify *knowledge gaps* in the clinical and nonclinical data:
• This may result in a recommendation for a juvenile monkey study
• Existing knowledge gaps, intended pediatric use, feasibility of study conduct and translatability of expected outcomes should be the primary drivers for study design

The overall risk assessment should be based on all available data for the molecule, the mechanism, and the intended pediatric population.

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Assessing Cognition in Nonhuman Primates using CANTAB

Robert W. Gould, Michael A. Nader

Abstract

The Cambridge Neuropsychological Test Automated Battery (CANTAB) was developed for the assessment of cognitive deficits in humans with neurodegenerative diseases or brain damage. More recently, CANTAB has been developed for use in preclinical research designed to understand the neurobiology associated with cognition and cognitive deficits. In this review, we briefly describe several of the most frequently used models of cognition that focus on executive function, working memory and behavioral flexibility. We also give examples of how these models can be used in nonhuman primates to examine several variables associated with drug abuse, including the long-term consequences of cocaine use, the ability of drugs to enhance cognition in monkeys with a long history of cocaine use and the combination of behavioral pharmacology, cognition and brain imaging to better understand the neurobiology of cognitive impairments.

1. Introduction

Cognitive performance affects all facets of human life. Cognition can be influenced by a number of conditions and a large number of factors contribute to cognitive deficits that are not well characterized in preclinical models. Many are compounded by genetic x environmental (x pharmacology) influences (Fig. 1). For example, delirium, an acute brain dysfunction, manifesting while in the intensive care unit (ICU) or during postoperative care has been linked with long-term cognitive impairments (LTCIs) resembling dementia, yet the underlying factors (e.g., stress, inflammation, age, cognitive reserve) leading to LTCIs are not well understood (Saczynski et al 2012; Pandharipande et al 2014). Increasing evidence supports a clear need to address the cognitive symptoms associated with neuropsychiatric and degenerative disorders as well. For example, currently available antipsychotic medications treat the positive symptoms of schizophrenia with little effect on the negative or cognitive symptoms (APA

2000) despite a relationship between cognitive improvement and overall functional outcome (Green et al 2004; Bobes et al 2007). Levodopa (L-DOPA), the most common treatment for motor symptoms of Parkinson's Disease, has been shown to have no effect or even exacerbate cognitive impairments in patients (e.g., Gotham et al 1988). Clearly, effects on cognition should be considered when evaluating overall efficacy of novel treatments for CNS-related conditions and preclinical studies are ongoing within these fields (e.g., Barch and Cesar 2012; Halliday et al 2014), but lie outside the scope of this review. Within the field of substance abuse, it has been hypothesized that neurobiological changes resulting in compulsive drug use have the added consequence of impairing decision making and inhibitory control, thereby contributing to continued drug abuse. One hypothesis is that treating the cognitive deficits will aid in maintaining abstinence. This review will describe preclinical animal models of cognition and will focus examples on one facet, that being cocaine-induced cognitive disruptions.

Factors negatively influencing Cognition		
Genetic/Physiological/Pathological	*Environmental*	*Pharmacologjcal*
AIDS	Diet/Nutrition	Antipsychotics
Acute Respiratory Distress Syndrome (ARDS)	Stress/anxiety	Antidepressants
Aging	Sepsis	Psychostimulants
ADHD	Sleep Deprivation	Alcohol
Alzheimer's Disease	Neuroinflammation	Opiates
Cancer	Traumatic Brain injury	Chemotherapy/Radiation
CNS lesions	Organic agents	
Dementia	PTSD	
Depression		
Eating Disorders		
Hypertension		
Menstrual Phase		
Parkinson's Disease	**Interactions or Comorbidities**	
Premature Birth	Stress/anxiety + Antidepressants/Anxiolytics PTSD + Depression + Drug use	
Schizophrenia	Schizophrenia + Antipsychotics	
Stroke		

Figure 1:
Cognition can be negatively influenced by a number of conditions, including interactions between gene, environment and pharmacology. Examples of such interactions are in the box.

1.1 CANTAB

The Cambridge Neuropsychological Test Automated Battery (CANTAB) apparatus was initially designed to study cognition in humans, but has been modified for use in animal models (rodents and nonhuman primates, NHPs). CANTAB programs consist of a series of visual and spatial tasks presented on a computer touchscreen designed to probe regional brain function by challenging specific cognitive components (Weed et al 1999). Recent applications include extension to NHPs to examine such variables as age, sex, disease progression, pharmacological manipulation, and CNS lesions of specific brain structures on task performance (Cirillo et al 1989; Dias et al 1996a,b, 1997; Voytko 1999; Weed et al 1999, 2008; Katner et al 2004; Porrino et al 2005; Schneider 2006; Hampson et al 2009; Taffe et al 2010; Crean et al 2011; Kromrey et al 2015). The focus of these studies has been on executive function. Broadly defined, executive function includes all processes involved in learning, monitoring and adapting to stimuli to produce complex, goal-oriented behaviors. Delineated cognitive domains include 1) updating, monitoring and adapting to cues relevant to a current goal and discarding/suppressing irrelevant information, 2) shifting, the ability to redirect focus between multiple modalities or tasks, and 3) inhibition, the ability to suppress or withhold a preplanned or impulsive response (see Miyake et al 2000; Beveridge et al 2008 for review). Numerous cognitive tests are designed to probe specific subsets within each of these domains and will be briefly described in this review.

1.2 Dopamine

While many neurotransmitters influence cognitive performance, this chapter will focus on the dopamine (DA) neurotransmitter system. The DA system is comprised of four neuronal pathways originating from the midbrain with projections to various brain structures (see Beaulieu and Gainetdinov 2011 for review). The nigrostriatal pathway originates in the substantia nigra pars compacta, innervates the dorsal striatum (caudate nucleus and putamen) and is involved in motor control. The mesolimbic pathway projects to the ventral striatum (nucleus accumbens), and other limbic structures including the amygdala, hippocampus, and cingulate gyrus and mediates actions related to reward, reinforcement, emotion, and motivation. The mesocortical pathway innervates cortical regions and is implicated in learning and memory. Lastly, the tuberoinfundibular pathway projects to the hypothalamus and influences anterior pituitary gland function. Dysregulation of the DA system through neurodegeneration or pharmacological insult can contribute to a number of disease states including Parkinson's

Disease, depression, attention-deficit/hyperactivity disorder, schizophrenia, and addiction (for reviews see Vallone et al 2000, Beaulieu and Gainetdinov 2011). Therefore, drug development strategies for these conditions, including addiction, focus on direct and indirect mechanisms that influence dopaminergic tone.

Within the DA system there are two families of DA receptors, the D1- and D2-like G-protein coupled-receptors, originally distinguished by their ability to stimulate and inhibit adenylyl cyclase activity, respectively. D1-like receptors are primarily located post-synaptically whereas D2-like receptors are located pre- and post-synaptically, functioning as autoreceptors as well as post-synaptic effectors. These receptor families are subdivided into D_1 and D_5 (D1-like) and D_2, D_3, and D_4 receptors (D2-like; subscripted numbers represent subtype). Both D_1 and D_2 receptors are predominately expressed in areas associated with the aforementioned DA pathways including the dorsal and ventral striatum, nucleus accumbens, substantia nigra, amygdala and frontal cortex with lesser expression in the hypothalamus, thalamus, cerebellum, and hippocampus. In contrast, D_4 and D_5 receptors are expressed in relatively low levels in various cortical and limbic regions with minimal expression in the striatum. DA D_3 receptor expression is limited to limbic regions. Also located on presynaptic DA nerve terminals in the striatum are DA uptake transporters (DAT) that function to transport synaptic DA intracellularly where it can be repackaged in vesicles by vesicular monoamine transporters or degraded by catechol-O-methyltransferase or monoamine oxidase (for reviews of the DA system see Vallone et al 2000; Beaulieu and Gainetdinov 2011).

1.3   Cocaine

Cocaine, which will be described in animal models later in this review, binds with near equal affinity to DA, serotonin, and norepinephrine transporters (DAT,SERT, NET, respectively; Ritz and Kuhar 1989; Bennett et al 1995) acutely elevating synaptic concentrations of all three monoamines (e.g., Di Chiara and Imperato 1988; Bradberry et al 1993; Florin et al 1994). Autoradiography studies in humans or monkeys with a cocaine self-administration history showed greater DAT (human, Little et al 1993; NHP, Letchworth et al 2001), NET (NHP, Macey et al 2003) and SERT (human, Mash et al 2000) binding compared to control groups, demonstrating potential neuroadaptations across each monoaminergic system as a result of cocaine exposure. Although multiple neurotransmitter systems contribute to aspects of the addiction cycle (i.e., craving, stress, reinstatement, cognitive disruptions) and are pursued as treatment targets for cocaine dependence, the reinforcing effects of cocaine are attributed

to elevated synaptic DA levels due to DAT blockade (Di Chiara and Imperato 1988).

1.4 NHPs

Although tasks from CANTAB have been modified for rat and mouse studies with an impetus for examining transgenic or knockout mouse strains (e.g., Bussey et al 2008; Horner et al 2013), we will provide examples from the use of NHPs. As it relates to translational research, NHPs are phylogenetically more related to humans and, along with baboons, Old World macaques (rhesus, *Macaca mulatta*, and cynomolgus, *M. fascicularis*) are the closest relatives of humans approved for biomedical research in the United States. Macaques have close homology to humans in terms of developmental and aging processes, neurotransmitter distribution, and complex social and cognitive behavioral repertoires (see Weerts et al 2007 and Nader et al 2012 for review). For example, humans and NHPs share greater than 95% overall gene homology and greater than 98% homology in monoaminergic transporters (Hacia et al 1998; Miller et al 2001). Further, documented differences in DA neuron innervation (Berger et al 1991; Joel and Weiner 2000) and affinity of DA for receptors between monkeys and rodents (Weed et al 1998) may be indicative of other differences in drug biodistribution, pharmacokinetic or pharmacodynamic interactions within the DA system (e.g., Lyons et al 1996; Roberts et al 1999; Lile et al 2003). As described below, the nicotinic acetylcholine system (nACh) is critically important in cognition and monkeys share similar nAChR localization, distribution and affinity states to humans (Cimini et al 1992; Han et al 2000; Quik et al 2000; Papke et al 2005). An additional advantage of NHP research is the ability for long-term studies and within-subject designs in controlled laboratory settings. Baseline behavioral, neurochemical, and hormonal measures can be correlated with changes following an experimental manipulation (e.g., chronic drug administration) while controlling for such factors as stress and nutrition over many years.

NHPs can learn complex cognitive tasks analogous or homologous to those administered to humans. Following a stable cognitive baseline, the effects of environmental or pharmacological manipulations can be evaluated. Such studies can be conducted to gain a better understanding of the acute and chronic drug effects on specific cognitive domains, or elucidate the neural substrates underlying cognitive performance through lesioning or imaging techniques (e.g., Dias et al 1996a,b; Porrino et al 2005; Gould et al 2012; Porter et al 2014). Neurocircuitry of the primate brain, specifically the prefrontal cortex, is similar to the

human brain (see Roberts et al 1996) and therefore provides relatively accurate extrapolation from animals to humans.

2. Models of cognition with a focus on NHPs

Cognitive performance affects all facets of human life and consequently models of human diseases that incorporate cognition as an endpoint provide an indication of treatment efficacy that is not usually considered in animal models (Fig. 2). With regard to drug abuse, the focus of this chapter, NHPs can be trained to perform tasks probing specific cognitive domains known to be impaired in human cocaine users. Importantly, tasks taken from human neuropsychological

Cognitive Assessment across Species			
Cognitive Domain	Sub-Domain	Human (touchscreen tasks)	Non-human Primate
Attention	Reaction Time (Attention)	Reaction time tasks (5-CSRRT)	Multiple Choice Serial Reaction Time Task (MCSRT)
	Sustained attention; Vigilance	Rapid Visual Information Processing (RVIP)	" "
		Continuous Performance Task (CPT)	" "
Learning and Memory	Associative Learning	Stimulus Discrimination (SD)	Acquisition of operant responding
	Associative learning (Gating/filtering)	Compound Discrimination learning (CD)	" "
Executive Function	Behavioral Inhibition	Reversal Learning (SDR)	" "
	Behavioral Flexibility	Attentional Set-shifting (ID/ED) Wisconsin Card Sorting Task	" "
	Impulsivity	Delay Discounting/ Probabilistic learning ("Impulsive Choice")	" "
	Response Inhibition/ Impulse control	Stop Signal Task (SST)	Stop Signal Reaction Time Task(SSRT)
	Response Inhibition	Go/No-Go	" "
Memory	Visual Working Memory	Delayed Match/Non-match to Sample (DMS/DNMS)	" "
	Visuo-Spatial Memory	Paired-Associates Learning (PAL); Matching sample-to-location	" " " "
	Visuo-Spatial Memory	Self-Ordered Spatial Search (SOSS)	" "

Figure 2:
Identical or homologous tasks implemented on touch-sensitive computer screens in human and non-human primates examining distinct cognitive domains. " " denotes identical tasks used in both human and non-human primates. See text for details.

batteries have been administered unaltered to monkeys using touch sensitive computer screens, while additional tasks have been modified for use in monkeys to examine similar cognitive domains as tasks administered in humans.

Despite similarities between human and NHPs and the numerous clinical studies demonstrating cognitive dysregulation in cocaine-experienced humans, few studies have examined the effects of cocaine on cognition in NHPs. Of these studies, the cognitive domains frequently assessed are associative learning, measured via a simple discrimination task, behavioral flexibility, measured via a reversal learning task, and measures of working memory, assessed by either a delayed alternation task or delay match-to-sample (DMS) task. These are briefly described below.

2.1   Executive function tasks

Cocaine users show impairments in shifting attention between tasks or behavioral flexibility as measured via set-shifting and reversal-learning tasks, respectively (Kubler et al 2005; Fillmore and Rush 2006; Beveridge et al 2008; Ersche et al 2008; Hanlon et al 2011). Both tasks probe different aspects of the shifting component of cognition. In the stimulus discrimination (SD) and discrimination reversal (SDR) task, two stimuli are simultaneously presented. In the simplest form, one stimulus is associated with a reward (S+) and the other stimulus has no reward (S-). In other versions, both stimuli are associated with a reward but with different magnitudes. In both scenarios, the animal must learn to discriminate between the two stimuli and choose the stimulus associated with the reward or the reward of greater magnitude. Using CANTAB, stimuli are presented using a touch-sensitive computer screen and monkeys respond by touching one stimulus or the other on the screen; correct responses result in delivery of food pellets or liquid reinforcers. Historically, the Wisconsin General Testing Apparatus (WGTA; e.g. Jentsch et al 2002) has been used to manually assess reversal learning. Two boxes are presented per trial, each with a distinct visual cue on top. A palatable treat is hidden within one of the two boxes and the monkey must discriminate between the two based on the visual cues. In either scenario, acquisition of the discrimination is operational defined as occurring, for example, when performance meets an experimenter-determined criteria such as 6 correct responses in a row, or a serial acquisition criteria such as 18 correct responses out of 20 consecutive trials. Following acquisition, the contingencies are reversed such that the previously reinforced (or higher magnitude of reinforcer) stimulus is no longer associated with reinforcement (or lower magnitude reinforcer); the S+ now becomes the S- and vice versa. Acquisition of

a simple discrimination can serve as a measure of associative learning while reversal learning provides an assessment of response inhibition, one aspect of behavioral flexibility.

Another measure of behavioral flexibility involves discrimination within and between multiple attentional sets. As before, a simple discrimination (i.e., SD) is acquired (e.g., two shapes, an S+ and S-). Second, a compound discrimination (CD) is introduced such that shapes are still discriminated but another attentional set (e.g., lines) overlay the shapes, and are not associated with any reward-related contingencies (serving only as distracter images); all four stimuli are randomly distributed independent of each other. Following acquisition of this stage, two new shapes and two lines are introduced but shapes continue to be the attentional set associated with reward. This stage employs an intra-dimensional (ID) shift, such that the previously established set remains in focus. The last stage is an extra-dimensional (ED) shift. One stimulus from the previously ignored attentional set (lines) now becomes the S+ and the previously reinforced set (shapes) serves as a distraction. While similar, a reversal task requires termination of a previously established stimulus-reinforcement association before the formation of a new association whereas set shifting requires a re-direction of attention, to a new, relevant attentional set and inhibition of a now irrelevant stimulus set. Importantly, elegant lesioning studies in monkeys align well with imaging studies in humans and have shown that different regions of the PFC are engaged during these tasks, providing a behavioral endpoint to assess regional brain function (see below).

Liu et al (2008) and Porter et al (2011) used a touch-sensitive computer screen to administer a discrimination and reversal task prior to and following various cocaine self-administration paradigms. In both studies, the discrimination contingencies were between high- and low-liquid reinforcers (1.0 ml and 0.3 ml H_2O) where a correct response required choosing the higher-magnitude reinforcer. SD/SDR performance occurred 72 hours following the last cocaine self-administration session and showed that initial SD acquisition (18 correct out of 20 consecutive trials) was hindered such that the SDR component could not be reliably examined (Liu et al 2008). Porter and colleagues (2011) showed that compared to a control group (n=6), initiation of cocaine self-administration did not impair initial percent accuracy (defined as the % of correct trials in the first 15 trials) on the SD but sustained accuracy (defined as 27 correct out of 30 consecutive trials) was impaired. Accuracy on the first 15 trials following reversal was also lower in the monkeys with a cocaine self-administration history compared to controls. There appears to be a rapid learning curve associated with the SD/SDR task in the simplest form (as described above) in that in

both male (Porter et al 2013) and female monkeys (Kromrey et al 2015) with a cocaine self-administration history, impairments in reversal learning are present upon the first exposure but upon repeated exposure performance between cocaine-exposed monkeys and cocaine-naïve monkeys is no longer different. Future studies wishing to examine reversal learning in monkeys across several time points may need to find additional ways to increase task difficulty or cognitive demand. For example, introducing distracter stimuli within each task can increase attentional demand and reduce baseline performance (e.g., Porter et al 2013).

Models of attention are also used to assess impulsivity – a critical phenotype in understanding drug addiction (Perry and Carroll 2008). Tasks measuring response inhibition include Go/No-Go tasks and stop-signal reaction time (SSRT) tasks. Both tasks involve training to associate responding to one stimulus and not responding upon presentation of a different stimulus. However, each task measures a slightly different aspect of motoric inhibition. The Go/No-Go task examines "action restraint". Trials involve either the presentation of a stimulus signaling responding (Go trial) or a stimulus signaling withholding a response (No-Go trial). Go/No-Go tasks may rely on an attentional component, since an individual must focus on multiple stimuli to signal and withhold a response. In contrast, SSRTs measure "action cancellation", the ability to inhibit a response during its execution. Similar stimuli signal emission of or withholding a response, yet the latter stimulus is presented at varying millisecond durations following the stimulus signaling responding. Eagle and colleagues (2008) provided an in-depth differentiation of the two inhibitory tasks. Importantly, cocaine users showed impaired inhibitory performance across both forms of inhibitory activity (Fillmore and Rush 2002; Kaufman et al 2003). One example from monkey studies, Liu et al (2009) designed a study in which conditions were similar to those used in humans and found that monkeys with a moderate cocaine self-administration history (~360 mg/kg cumulative intake) showed increased impulsivity, represented by long SSRT measures, following 18 months of abstinence from cocaine self-administration.

2.2   Working memory (updating) tasks

Working memory can be examined in animals using visual or spatial cues. Classic working memory tasks include delay match- and non-match-to-sample tasks (DMS or DNMS). In both tasks, a stimulus is presented to the animal that must be retained across a variable-delay interval. Following this delay, the animal must select from an array of stimuli either the same stimulus (match) or the op-

posite stimulus (non-match) presented prior to the delay. Tasks assessing spatial memory are similar except that instead of recalling a specific stimulus, the location of the stimulus on a screen must be recalled. Similarly, in a delay-alternation task, two stimuli are presented and each subsequent correct response must be made on the stimulus not touched in the preceding trial. In all measures of working memory, increasing the delay value or the number of distracter images increases the cognitive demand; these variables are manipulated often on an individual basis to engender demand-dependent curves such that short delays/few distracters produce a high percentage of correct responding and longer delays/ more distracters produce a lower percentage of correct responding.

In one study examining working memory performance in NHPs, two rhesus monkeys with cocaine (average intake 360 mg/kg) and cocaethylene histories (a metabolite produced by the interaction of cocaine and alcohol; average intake 90 mg/kg), self-administered 0.5 mg/kg/injection cocaine (max 3.0 mg/kg cumulative daily intake) once weekly (Liu et al 2008). These monkeys were trained on a delay alternation task. Compared to controls, monkeys with a cocaine self-administration history required a greater number of trials to acquire this task (80% accuracy with a 0-sec delay). There were no initial differences over 10 sessions when delays were introduced (0-10 sec) but over 17 weeks of assessment the cocaine-naïve monkeys improved at a significantly greater rate than the two monkeys continuing to self-administer cocaine (Liu et al 2008). In another study by the same group, eight monkeys self-administered the same dose as above 4 days/week and visual working memory was assessed weekly using a DMS task, following 72 hours of abstinence (Porter et al 2011). Compared to a control group (n=6), percent accuracy at the longest delay was lower during the first 4 assessments (1 month), but tolerance developed to the disruptive effects of cocaine. In both of these studies, cocaine was intermittently self-administered and 72 hours elapsed before each cognitive assessment.

We have extended this characterization to monkeys with an extensive cocaine self-administration history (Gould et al 2012). In that study, adult male rhesus monkeys with an ~ 5 year cocaine self-administration history and age-matched controls (n=4/group) performed cognitive tasks in morning sessions and self-administered cocaine or food in afternoon sessions (5-7 days/week). When studied under a DMS task, there were no baseline performance differences in working memory between groups. When studied under the SD/SDR and ID/ED tasks, measures of behavioral flexibility, these same cocaine-experienced monkeys required significantly more trials and committed more errors in acquiring the reversal stage and in acquiring the ED stage compared to controls (Gould et al 2012). These findings suggest that cocaine exposure does not decrease all

aspects of cognitive function, but may be task specific. In an effort to determine if there were conditions in which cocaine would affect working memory, the cocaine-experienced monkeys were given access to higher doses of cocaine in daily self-administration sessions. Under these conditions, DMS performance was initially disrupted, but tolerance developed. Finally, when these monkeys were studied during abstinence from cocaine, DMS accuracy increased significantly by Day 30 of abstinence, while performance of cocaine-naive monkeys was unchanged. The positive outcome during abstinence is highly encouraging and suggests that behavioral strategies, perhaps in combination with pharmacotherapies, may enhance cognition and treatment outcomes.

Decision-making tasks in humans, such as the Iowa Gambling Task (IGT) require concurrent use of updating, shifting, and inhibitory domains. In this task, participants must choose between 4 decks of cards that are associated with various advantageous and disadvantageous scenarios (monetary gains and losses) each occurring with different frequencies. Participants develop a choice selection strategy for perseverating on or choosing between decks based on updating information from previous trials. Not surprisingly, cocaine users show impaired performance on this task compared to controls (e.g., Bechara et al 2000; Verdejo-Garcia et al 2007). Although the CANTAB platform offers additional tasks of increasing complexity that incorporate multiple cognitive domains, including the paired-associates learning and self-ordered spatial search tasks (e.g., Weed et al 1999; Taffe et al 2002), neither (to our knowledge) have been conducted in monkeys preceding or following a cocaine self-administration history. It is important to emphasize that in an effort to more closely model human cognitive testing, continued efforts are necessary to develop NHP analogs of more complex tasks used in human studies that probe multiple cognitive domains simultaneously.

Before closing this section, it is important to note that the majority of studies described above utilized male monkeys. A recent study found that cocaine history also impaired cognitive performance in female cynomolgus monkeys (Kromrey et al 2015). In that study seven female monkeys with an extensive history of cocaine self-administration, but had been abstinent for at least 2 months, were compared to nine cocaine-naïve control monkeys on two cognitive tasks – SD/SDR and DMS. Cocaine-naïve monkeys required fewer total trials and made fewer errors and omissions before acquiring the SD and SDR tasks compared to monkeys who had previously self-administered cocaine. This outcome is similar to what was observed in male monkeys from the same laboratory (Gould et al 2012). However, in the female monkeys, this cognitive impairment dissipated over several months of exposure to the task. As was noted

with male monkeys, there were no differences between cocaine-experienced and cocaine-naïve female monkeys on DMS task performance. While it appears that baseline performance on these two cognitive measures are similar in male and female monkeys, it remains to be determined whether there are sex differences in response to pharmacological and environmental (e.g., stressors) challenges. Outcomes from these types of studies are needed to support the strategy of personalized medicine in addiction.

3. Understanding the neuropharmacology of cocaine-induced cognitive impairments using NHPs

In addition to neuropsychological test batteries administered to current or recently abstaining cocaine users, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies have provided new opportunities to examine specific brain function underlying specific cognitive domains. Recent fMRI studies have shown functional deficiencies associated with impaired executive function compared to control groups. Various regions of the prefrontal cortex are typically activated (e.g., dorsolateral, ventromedial, or orbital prefrontal cortex) dependent on the cognitive domain being assessed. Consistently, cocaine users showed less activity in the anterior cingulate cortex (ACC) and relevant regions of prefrontal cortex (PFC), notably the dorsolateral PFC (dlPFC) and orbital PFC (orbPFC) during tasks measuring updating, shifting, and inhibitory domains (Fillmore and Rush 2002; Bolla et al 2004; Hester and Garavan 2004; Kubler et al 2005; Goldstein et al 2007, 2010; Woicik et al 2009).

A particular advantage of PET is the ability to study receptors *in vivo* in longitudinal studies. More specifically, PET imaging techniques allow an examination of neuronal function and regulation through utilization of radiolabelled molecules with affinity and selectivity for specific neuronal structures (mitochondria) or proteins (e.g., neurotransmitter receptors or transporters). PET imaging techniques in NHP models of addiction have substantially increased our understanding of the effects of cocaine on the brain and the malleability of brain function through pharmacological and environmental factors (see Nader et al 2008; Howell and Murnane 2011, Murnane and Howell 2011; Nader and Banks 2014 for reviews).

Cognitive deficits associated with cocaine use may perpetuate the cycle of drug use and increase relapse through 1) dysregulation in pathways controlling behavioral inhibition and/or impulsive behavior, and 2) predisposing abstaining

drug users to failure in behavioral treatments in which cognitive-based approaches are designed to educate and modify maladaptive behaviors (cf. Rogers and Robbins 2001). In fact, neurobiological and behavioral measures obtained from cocaine-dependent treatment seekers just prior to treatment initiation have been directly linked to rates of attrition and treatment success (e.g., Teichner et al 2001; Turner et al 2009; Martinez et al 2011). Therefore, using PET imaging to better understand the neurobiological alterations underlying impaired executive function as a result of cocaine self-administration and the persistence of these effects is integral in developing effective treatment strategies.

3.1 Measuring brain function using positron emission tomography (PET) imaging in NHPs

One application for using animals and cognitive testing is to determine the underlying neural substrates mediating cognitive performance. For example, Dias and colleagues (1996a,b, 1997) lesioned either the dlPFC or orbPFC in monkeys to determine the involvement of each brain region in acquiring set-shifting and rule-reversal tasks. Their findings suggested that chemically induced lesions to the orbPFC selectively inhibited reversal learning while lesions to the dlPFC selectively inhibited set-shifting behavior, results that were similar to cognitive deficits seen in human patients with natural lesions to similar brain regions. These studies highlight similar neurobiological substrates underlying specific cognitive function between NHPs and humans (Dias et al 1996a,b, 1997). Similarly, transection of the temporal stem, the white matter tract connecting the temporal lobe to the thalamus and PFC impaired working memory in monkeys performing a DMS task implicating the temporal cortex in this type of cognitive performance (Cirillo et al 1989).

However, lesioning studies only implicate the involvement of one component of a neural pathway in mediating a specific behavior. PET, SPECT, and fMRI techniques can provide a broader assessment of CNS function, including multiple regions within the brain that are active during cognitive performance relative to a specific behavior. In this section, we describe a study involving PET imaging and the PET tracer [18F]-fluorodeoxyglucose (FDG), which is an analog of glucose, and can be used to examine metabolic rates of cerebral glucose utilization (MRglc). The primary energy source in the brain under normal circumstances, glucose is transported into cells to restore and maintain chemical gradients. Increased rates of FDG uptake reflect increases in local energy use, representing a measure of functional activity. These methods are intended for the evaluation of manipulations that occur over relatively short time frames

(e.g., drug administration or brief behavioral task) with changes in MRglc calculated by comparison to scans obtained during baseline conditions (see Henry et al 2010). For example, Porrino and colleagues (2005) have used FDG and PET imaging to measure MRglc in order to elucidate the substrates underlying working memory as assessed via a delay match-to-sample task. During trials with a long delay compared to no delay, glucose utilization was higher in the temporal cortex as well as the PFC and limbic regions. Recently, we extended this characterization of brain glucose utilization during cognitive performance of monkeys to include cocaine self-administration (Gould et al 2012).

Earlier in the chapter, we described cognitive performance of these monkeys under three tasks: SD/SDR, ID/ED and DMS (Gould et al 2012). For the imaging studies, these monkeys were re-tested on the ID/ED set-shifting task while using a novel set of stimuli. These monkeys performed cognitive tasks in the morning and either self-administered cocaine or responded under an identical schedule of food reinforcement (control groups). For these studies, each monkey underwent two FDG-PET studies associated with cognition: (1) a baseline (BL) session to control for visual-motor activity and (2) during an ED task. The order of PET studies was counterbalanced between BL and ED tasks and separated by at least 2 weeks. As hypothesized, the investigators noted modest differences in glucose utilization between cocaine-experienced and cocaine-naïve groups during the BL condition. During the ED task, there were several significant differences between groups noted (Fig. 3). The cocaine-experienced monkeys showed greater glucose utilization in the left superior occipital gyrus, while there were several regions in which increased glucose utilization was observed in the cocaine-naive monkeys (Fig. 3). The cognitive deficits noted between cocaine-experienced and cocaine-naïve monkeys were associated with differences in glucose utilization assessed via FDG-PET. In contrast, a more recent study examined glucose utilization in monkeys with a cocaine self-administration history when performing a DMS task. Although performance was not different between cocaine-experienced and cocaine-naïve monkeys, cocaine-experienced monkeys showed less dlPFC and greater cerebellar MRglc compared to control monkeys, suggesting a potential compensatory brain function (Porter et al 2013). It is believed that understanding the neuropharmacological consequences of long-term drug use will lead to novel treatment strategies for cocaine addiction.

As described above, cocaine blocks DA reuptake and the elevated concentrations of DA bind to two families of DA receptors, D1- and D2-like receptors. Both D1- and D2-like receptors have been implicated in mediating executive function. However, evidence supports a dichotomous relationship regarding
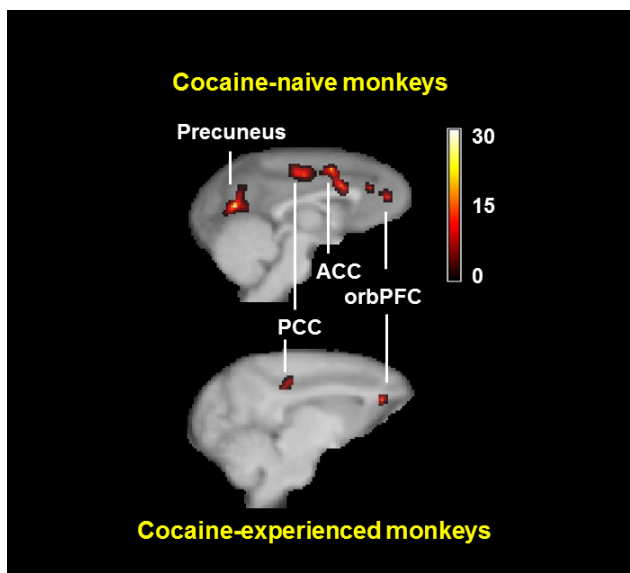
Figure 3:
Areas of increased glucose utilization during an extradimensional shift. Group data from whole-brain analyses showing areas where cocaine-naive monkeys (top) and cocaine-experienced monkeys (bottom) showed significantly greater glucose utilization during the initial response to an EDS compared to their respective baseline measures. Abbreviations: orbPFC – orbital prefrontal cortex; ACC – anterior cingulate gyrus; PCC – posterior cingulate gyrus. See Gould et al (2012) for complete details.

subtype-selective dopaminergic regulation of cognition and this relationship appears to be task dependent. D1-like receptors appear to be integral for working memory, (cognitive stability) whereas D2-like receptors appear to mediate higher order tasks requiring behavioral flexibility (for review see van Schouwenburg et al 2010). In general, D1-like agonists improved spatial working memory in monkeys but with a narrow therapeutic window (see Mehta and Riedel 2006 for review). In contrast, D2-like receptor availability directly correlated with performance on a reversal-learning task in monkeys (Groman et al 2011). These examples support the notion that dysregulation within the DA system may be an underlying cause of cognitive impairments associated with cocaine use.

Drugs directly targeting the DA system have largely been unsuccessful in maintaining abstinence in treatment-seeking cocaine users. Therefore, an indirect mechanism through which to modulate DA function is proposed for further study. In humans, nicotine and nicotinic acetylcholine receptor (nAChR)

compounds can increase neural function and improve cognition (e.g., Lawrence et al 2002; Rezvani and Levin 2001). Of clinical relevance, nicotinic agonists can indirectly stimulate DA function which may improve executive function in treatment-seeking cocaine users and improve the success of behavioral treatment strategies. In two studies by Gould et al (2011, 2013), the effects of nAChR compounds were assessed for their ability to influence the abuse liability of cocaine using cocaine self-administration, cognition using the CANTAB battery, and nicotinic AChR availability using PET imaging.

3.2 Nicotine receptor-based measures of brain function using PET imaging in NHPs

Within the CNS, the ACh neurotransmitter system serves a neuromodulatory role to influence signaling of other neurotransmitters such as DA. There are two primary ACh projection pathways within the CNS. Cholinergic basal forebrain projections to the thalamus and cortex play a role in arousal, attention and memory while midbrain cholinergic neurons projecting to the ventral tegmental area (VTA) and cholinergic interneurons in the striatum can directly influence DA neurotransmission (see Perry et al 1999; Exley and Cragg 2008 for review).

Both nicotinic (ligand-gated ion channels) and muscarinic (G-protein coupled) acetylcholine receptor subtypes can influence DA function and cognition but the focus within this review will be on nAChRs. To our knowledge, no human study has investigated nAChR distribution associated with cocaine exposure using PET imaging. Adinoff and colleagues (2010) examined regional cerebral blood flow (rCBF) in cocaine users. Following pharmacological challenges, alterations in rCBF were present between cocaine users and non-drug controls in regions underlying learning and memory including the hippocampus, although similar differences were seen with the muscarinic AChR antagonist scopolamine and the nonselective muscarinic and nicotinic AChR agonist physostigmine. While this study implicated changes in the ACh system, per se, effects could not be directly attributed to nAChRs exclusively. Further, cocaine users in this study were also nicotine-dependent, suggesting differences were not specific to the effects of cocaine (Adinoff et al 2010).

In the final study to be described, Gould et al (2013) examined nAChR availability in monkeys with a cocaine self-administration history and cocaine-naïve monkeys using [11C]-nicotine and PET. For this study, nAChR availability was determined in four adult male rhesus monkeys with an extensive cocaine self-administration history (~6 years, mean intake, 1463 mg/kg) and compared to

age-matched cocaine-naive control monkeys (n=5). The cocaine-experienced monkeys had significantly higher [^{11}C]-nicotine receptor availability in the hippocampus compared to cocaine-naive monkeys. Surprisingly, no other regions were significantly different between groups. Since [^{11}C]-nicotine is a nonselective nAChR ligand, subtype selective alterations following cocaine self-administration are not known, but this study demonstrated in a preclinical model that cocaine may alter nAChR-related neurobiology.

Nicotinic AChR agonists (e.g., nicotine, cytosine, varenicline) have been shown to increase DA release in the striatum and in the PFC determined in both *in vitro* and *in vivo* studies (Zhou et al 2001; Zoli et al 2002; Rice and Cragg 2004; Rollema et al 2007; Abin-Carriquiry et al 2008; Chan et al 2007; Livingstone et al 2009). Nicotinic AChRs are comprised of 5 subunits of homogenous (primarily containing α7 subunits) or heterogenous (α1-10; β1-4) subunits of which the homomeric α7 receptors and the α4β2* receptors are the most abundant within the CNS (Corringer et al 2000; Quik et al 2000; Le et al 2002). Differential localization and distribution with the CNS support different influences on DA function (see Dani and Bertrand 2007; Picciotto et al 2008 for reviews). Therefore, stimulation of various nAChR subtypes may differentially affect DA function, including cognition (e.g., Kadir et al 2006, 2007).

As noted above, one novel treatment approach that may increase success of abstinence in treatment-seeking cocaine users is improving the cognitive deficits associated with long-term cocaine use or subsequent abstinence. In these same cocaine-experienced and naïve monkeys described above, nicotine, varenicline, a low-efficacy α4β2*-selective agonist, and PNU-282987, a novel, high-efficacy α7-selective nAChR agonist all improved working memory performance at the longest delay of a DMS task (Gould et al 2013). These studies are important in demonstrating pharmacological enhancement of cognition in NHP models of cognitive deficits associated with long-term cocaine use. Importantly, clinical studies are now reporting similar results (Mahoney et al 2014). Interestingly, the same doses that improved memory in the DMS disrupted performance on an SD/SDR task in cocaine-naïve monkeys only, without affecting performance in the cocaine-experienced monkeys. Together these studies reinforce the fact that pharmacological agents may engender different effects on different cognitive domains in subjects based on behavioral or pharmacological history. This reiterates the importance of examining putative pharmacotherapies in several preclinical models related to clinical diseases. Lastly, these data reiterate the need to assess cognitive performance across multiple domains as provided within the CANTAB battery.

4.   Conclusions

Despite decades of research, there still remains no FDA-approved treatment for assisting cocaine users to remain abstinent. However, a great deal has been learned from clinical and preclinical studies regarding immediate and long-lasting neurobiological, behavioral, and cognitive changes following cocaine use that can help direct treatment approaches. Importantly, evidence suggests that some of the neurobiological changes and cognitive impairments may dissipate with increasing periods of abstinence (e.g., Nader et al 2002, 2006; Hanlon et al 2011; Gould et al 2012; Potvin et al 2014; Vonmoos et al 2014; Kromrey et al 2015). The etiology of addiction is complex and successful treatments will be neither simple nor singular. Targeting multiple aspects of addiction is more efficacious than any one facet. For example, combination behavioral strategies improved treatment retention or sustained abstinence to a greater extent than single methodologies. Although pharmacotherapies are largely unsuccessful administered alone, evidence suggests that drug treatments can complement existing behavioral strategies to increase overall treatment retention and sustained abstinence. These behavioral strategies involve improving cognitive performance and include methods of cognitive behavioral therapy (CBT) and contingency management (CM). From the limited reviews above of preclinical and clinical literature describing cognitive deficits and their neurobiological correlates, one added therapeutic approach should be to enhance cognitive function in an effort to improve success of these behavioral modification strategies (Schmitz et al 2009). We will close this review with a few examples of combined behavioral and pharmacological treatment approaches.

In preclinical models, antidepressants have shown success in reducing cocaine related-behaviors. However, in methadone-maintained cocaine users, the antidepressant fluoxetine (Grabowski et al 1995) and desipramine (Kosten et al 1992) did not significantly reduce the percent of drug-free urine samples compared to control groups when treatment included CBT sessions alone. However, when these pharmacotherapies were administered in combination with CBT + CM techniques, both fluoxetine (Schmitz et al 1998) and desipramine (Kosten et al 2003) resulted in fewer drug-positive urine samples across the treatment period. These results are similar to other studies examining antidepressants in combination with CM techniques including disulfiram (Petrakis et al 2000), citalopram (Moeller et al 2007) and bupropion (Poling et al 2006). It is noteworthy that the efficacy of antidepressant drugs to improve treatment success are not specific to one mechanism and are similar with the tricyclic antidepressant (desipramine), selective SSRIs (fluoxetine, citalopram) and the mixed DAT/SERT/NET inhibitor (bupropion) when combined with CM strategies.

Similarly, CM strategies and indirect DA agonists are more efficacious in reducing drug-free urine samples compared to DA agonists alone or DA agonist + CBT. In a recent meta-analysis examining 26 clinical studies that assessed indirect DA agonists in cocaine or cocaine/opiate dependent patients in combination with various behavioral treatments, *d*-amphetamine and bupropion were the only two drug treatments that trended toward an overall significant effect on the primary outcome measure of percent drug-free urines (Perez-Mana et al 2011). Of these 26 studies, only three implemented CM strategies. Although cognitive testing was not included in the above studies, more recent studies have shown that modafinil and methylphenidate, two drugs that can increase DA function have shown cognitive enhancing effects in cocaine users (Kalechstein et al 2013; Moeller et al 2014).

These results highlight the importance of implementing combined behavioral and pharmacological strategies to treat drug dependence and that drugs that enhance cognition may enhance success of these behavioral approaches. It has been hypothesized that CM strategies provide more immediate positive reinforcement compared to CBT in which the emphasis is long-term abstinence through behavioral modification, and thus may reduce the relative reinforcing effects of the alternative, drug reinforcer, resulting in longer rates of abstinence. While research is ongoing to design the most effective behavioral treatment platform to pair with pharmacological agents, presently CM appears to be the most efficacious when implemented in conjunction with CBT and pharmacological adjuncts (e.g., Carroll et al 2004; Schmitz et al 2008, 2010). The goal of this chapter was to describe research utilizing the CANTAB cognitive assessment platform in NHP models of cocaine abuse. Cocaine self-administration in NHPs produces similar cognitive impairments, and neurobiological alterations as those seen in current or recently abstaining cocaine users. Future research will continue to examine a novel pharmacological approach, cognitive enhancement, and the use of *in vivo* imaging to study brain changes that may lead to improved behavioral and pharmacological strategies to aid in treatment of cocaine dependence.

5. Acknowledgements

6. References

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Potential of Genetically Modified Nonhuman Primate Models for Biomedicine

R. Behr

Abstract

The spectrum of model organisms in biomedical and preclinical research is very broad. It ranges from yeast to non-human primates (NHP). Due to their close phylogenetic relationship to humans, which is reflected by generally very similar anatomy and physiology, NHP are particularly important in preclinical research. Therefore, NHP are also very important in preclinical safety and efficacy testing of novel therapeutic approaches. Many human disorders and clinically relevant conditions, however, do not occur spontaneously in NHP (or were not yet identified in there). Therefore, recent groundbreaking advances in the genetic modification of NHP are of utmost significance. They offer the option to generate accurately designed NHP models of human genetic disorders. It will be possible to induce gain of function and loss of function mutations. This will allow modelling a variety of medically and socio-economically highly relevant diseases including neurological, immunological and metabolic disorders. The potential of genetically modified NHP models for biomedicine is considered immense.

1. Organisms in biomedical research: why using NHPs?

The spectrum of model organisms in biomedical and preclinical research is very broad. It ranges from yeast, which is an extremely useful model organism for instance in cell cycle, cancer (Matuo et al. 2012) and metabolism research (Natter and Kohlwein 2013) to non-human primates (NHP). Well-established model organisms are also the nematode *Caenorhabditis elegans* (Alexander et al. 2014), the fruit fly *Drosphila melanogaster* (Prussing et al. 2013, Smith et al. 2014) and "lower" vertebrates like the zebra fish (*Danio rerio;* (Newman et al. 2014, Santoro 2014). If specific mammalian characteristics are studied, mammalian species like the mouse (Webster et al. 2014), dog (Davis and Head 2014) or pig (Suzuki et al. 2011, Wolf et al. 2014) appear particularly attractive. NHP belong to the same mammalian order as humans and share many charac-

teristic biological features with humans and are therefore, from the phyloge-netic perspective, the most favorable animal models (Finstermeier et al. 2013, Phillips et al. 2014). Besides all valuable cell and animal models currently available and all prospective *in vitro* and *in silico* models, NHPs will remain important, probably even irreplaceable models in specific research areas (Phil-lips et al. 2014) which include neurosciences, behavioral biology, immunology / infection biology, reproductive and stem cell biology. In preclinical research, NHP will be of high relevance for the investigation of pathological processes during disease initiation and progression and also in preclinical safety and ef-ficacy testing of novel therapeutic approaches (Berman et al. 2014).

2.   Genetic modification of model organisms: general considerations

Many human disorders and clinically relevant conditions do not occur sponta-neously in model organisms (or were not yet identified in these species). This includes diseases with a genetic cause like familial Parkinson's disease (PD, Trinh and Farrer 2013), Huntington's disease (HD, Goldberg et al. 1994), and the Rett syndrome (Dragich et al. 2000). Therefore, genetic modification is of-ten a prerequisite to establish use- and meaningful model systems. These can be either generated by introducing gain of function mutations or loss of function mutations.

In general, there is a correlation between the biological complexity of the or-ganisms and the experimental complexity to introduce a genetic modification. Yeast strains, for instance, can be easily modified (Forsburg 2001); the same is true for the genomes of the invertebrates *C. elegans* (Baylis and Vazquez-Manrique 2011) and Drosophila (Lin et al. 2014). In contrast, genetic modifi-cation of mice, although routine in numerous biomedical research institutions, is more complex and laborious (even in times of RNA-guided nucleases, see below) than the genetic modification of invertebrates. Even more complex and time consuming is the genetic modification of large model organisms like the dog (Chastant-Maillard et al. 2010, Lee et al. 2014) or the pig (Hauschild et al. 2011, Whyte and Prather 2011). The major determinants of the complexity of genetic modifications are (i) technical aspects, (ii) accessibility of the (germ-line) cells that have to be modified and (iii) the biological generation time of the species. The (technical) options to introduce the gene modifying system into the cells include (self-executing) viral vectors and direct micro-injection into the respective cell type. Concerning accessibility of the cells, there are tremen-dous differences between species: yeast cells for example are very easily acces-sible, while access to monkey oocytes or embryos requires considerable efforts.

The generation time strongly influences the period of time until a reasonable number of genetically modified organisms is available. It ranges from less than two hours in yeast (Herskowitz 1988) to years in large animal models. Hence, when a model organism is selected to answer a research question, many parameters have to be taken into account.

3. Genetic modification of NHP: the reproductive biology perspective

Generally, there are two fundamentally different approaches to generate genetically modified NHP: (i) local application of the genetic modifiers to individual animals or (ii) genetic modification of oocytes or preimplantation embryos resulting in transgenic animals that have the potential to transmit the introduced modification via transgenic gametes to their offspring. This approach provides the highly attractive option to establish a cohort of animals carrying the same genetic modification by breeding.

Local *in vivo* genetic modification is mostly based on the use of (lenti-) viruses which can actively enter the cells and release their modifying genetic material into the cells at the injection site (Dissen et al. 2009). For instance, injections of transgene-expressing viruses in specific brain regions result in animals which exhibit local transgene expression only in the respective areas of the brain (Colle et al. 2010, Kirik et al. 2003, San Sebastian et al. 2013). This approach is rapid, technically relatively simple and may mimic endogenous expression patterns of RNAs and proteins of interest. However, one major disadvantage is the variability between individual modified animals since there might be difficulties in targeting exactly the same brain area in individual animals. Also the number of viral integration sites differs between individual cells in an animal. Because of the local restriction it is further not possible to mimic a human disease state going along with ubiquitous presence of a genetic mutation. Finally, random integration of a viral DNA into the target cell genome may result in adverse insertion mutagenesis.

In contrast to the local genetic modification, the genetic modification of oocytes or preimplantation embryos provides the highly attractive option of the long-term establishment of animal cohorts with identical and inheritable genetic modifications. The reduced experimental variability of studies utilizing bred offspring with a defined genetic constitution of the animals is of invaluable importance with regard to controlled randomized preclinical studies in NHP. A prerequisite for this strategy, however, is an *in vitro* fertilization / embryology /

micromanipulation laboratory where manipulation of germline cells or embryos is possible. The germline includes all cells that have the potential to transmit (at least half of) their genetic information to the next generation. Mouse embryonic stem (ES) cells for instance can be genetically modified *in vitro* and then injected into a preimplantation blastocyst, where they integrate into the inner cell mass and form a chimeric embryoblast with the endogenous embryonic cells. Eventually, after retransfer of the chimeric embryo to a surrogate mother and its development to term, the genetically modified cells may have contributed to the testicular or ovarian germ cell pool of the developing animal. This offers the chance of transmitting the modification to the next generation by natural breeding. However, the injection of NHP ES cells into pre-implantation embryos did not result in chimeric NHP embryos so far (Tachibana et al. 2012). To achieve genetic modifications of NHP, developmentally competent oocytes or pre-implantation embryos were directly manipulated (Chan et al. 2001, Liu et al. 2014, Niu et al. 2014, Niu et al. 2010, Sasaki et al. 2009, Wolfgang et al. 2001) instead of cultured ES cells as in mice. Importantly, irrespective of the applied method and irrespective of the stage and type of germline cell that was modified: once transmitted through the germline, the genetic modification is usually stably and uniformly present in all cells of an individual that develops from a genetically modified gamete.

This review focuses on approaches targeting the germline of NHP.

4. Genetic modification of NHP: the functional and molecular biology perspective

4.1 Lentiviral transgenesis is successful, but also has some limitations

Functionally, there are two different categories of genetic modification: gain of function and loss of function mutations. Gain of function mutations can be achieved by introduction of additional genetic information in order to express an additional gene or protein on the wildtype genetic background or by targeted genetic modification of an endogenous gene in order to modify the biological function of the corresponding endogenous protein. Loss of function mutations can be obtained by the functional ablation of endogenous genes resulting in no or non-functional protein.

Introduction of additional genetic information can be achieved by lentivirus-mediated approaches. After a transgene has been introduced into the viral genome, the viruses can be injected under the zona pellucida of ooyctes or non-

hatched preimplantation embryos. Upon transduction of the oocyte or the blastomeres, the viruses integrate randomly, stably and irreversibly into the host cell genome and initiate expression of transgenes. Different studies have shown the feasibility of this technique (Chan et al. 2001, Niu et al. 2010, Sasaki et al. 2009). Importantly, also germline transmission of the transgenes was demonstrated (Sasaki et al. 2009). Viral transgenesis allows the modelling of diseases which are caused by dominant mutations like Huntington's disease (Yang et al. 2008; for details, see below). However, there are also clear limitations associated with the use of lentiviruses: as already mentioned, these viruses randomly integrate into the genome, potentially causing adverse mutations. Since it is impossible to target specific sites in the genome, targeted loss of function studies and the site-specific introduction of mutations are not feasible using conventional viral transgenesis. Additionally, some viral promoters are prone to epigenetic silencing. This may result in a situation where the transgene is present but not expressed.

4.2 CRISPR/Cas9: a pioneering technology enables precise *in vivo* genome modifications

A real breakthrough in gene modification technology was the invention of the TALEN and the CRISPR/Cas9 technology. Both technologies take advantage of the fact that DNA-nucleases can be guided to specific sites of the genome which can be predefined by the study designer. This allows the site-specific introduction of DNA double strand breaks. Due to an inaccurate repair process, the genetic information is often not restored accurately and deletions of only one or a few base pairs can result in open reading frame shifts leading to loss of function mutations. Hence, a functional gene knockout can be obtained. Site-specific gain of function mutations can also be introduced by the CRISPR/Cas9 system by simultaneous delivery of DNA oligonucleotides or whole open reading frames of genes flanked by homology arms. With a certain probability, the endogenous DNA is replaced by the mutated exogenous DNA during the DNA repair process. Importantly, these techniques allow the targeted genetic modification also of animal species where ES cell technology is not available. In case of mammals, the only prerequisite for the application of the TALEN or CRIPSR/Cas9 technology is the availability of reproductive biology techniques such as oocyte / embryo retrieval, oocyte / embryo micromanipulation for the delivery of the nucleases and retransfer to the modified embryos to surrogate mothers.

Table 1:
Comparison of lentiviral transgenesis and the CRISPR/Cas-mediated approach for genetic modification of non-human primates.

	Lentiviral transgenesis	CRISPR/Cas (RNA-guided nucleases)
Entry into cells	Active	Passive (injection into (fertilized) oocytes)
Site-specificity of the genetic modification	None (risk of random insertion mutagenesis)	Yes (off-target effects cannot be excluded, but were not found so far)
Type of modification	Overexpression of additional proteins	Targeted deletion of genes, invention of point mutations, targeted insertion of DNA sequences
Preparation of vector	Relatively laborious Safety issues	Easy, no safety issues

TALENs (Transcription activator-like effector nuclease) consist of two important functional domains: a DNA binding and a nuclease domain. The DNA binding domain can be designed to bind to a defined DNA sequence and guides the nuclease to the gene of interest within the living cell.

Although the TALEN technology was a major advance in the field of genetic modification of mammals (Liu et al. 2014, Wefers et al. 2013), it was soon overrun by an even more convenient and more straightforward technology. The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas technology (Hsu et al. 2014, Sampson and Weiss 2014) revolutionized the field of genetic modification in living animals including mammals (e.g. Niu et al. 2014, Wang et al. 2013, Yang et al. 2013). In contrast to TALENs, where the DNA sequence of interest is recognized by a protein harboring a DNA binding domain, the CRISPR/Cas technology is based on guidance of the nuclease to the target site directly by a short RNA which is complementary to the target DNA (for review of the CRISPR/Cas9 technology see Hsu et al. 2014, Sampson and Weiss 2014). Very importantly, this technique was shown to allow the simultaneous introduction of multiple modifications (Niu et al. 2014, Yang et al. 2013) offering options that could not be foreseen only a few years ago. It is a quick, cheap and relatively simple technology which allows the predefined introduction of loss of function and gain of function mutations in animals. Due to its simplicity and versatility it can be applied to virtually any species allowing the targeted genetic modification also of animal species where ES cell technology

is not available. In fact, this technology has been shown to work efficiently in different mammalian species like mouse, rat, rabbit, pig and NHP (Honda et al. 2014, Liu et al. 2014, Nakamura et al. 2014, Wang et al. 2013, Whitworth et al. 2014). The technological progress in the field of CRISPR/Cas is currently overwhelming and suggests that the methodological repertoire for the genetic modification of mammals will further expand within the next few years (Hsu et al. 2014).

5. Genetic modifications of NHP: current published state

5.1 Lentiviral vector-based overexpression of transgenes

In 2001, in two concurrent studies, the cDNA encoding enhanced green fluorescent protein (EGFP) was transduced into mature rhesus monkey oocytes which were subsequently fertilized *in vitro* and then transferred to surrogate mothers. The study of Chan and colleagues was groundbreaking since it showed for the first time that general – and not only local – genetic modification of NHP was possible (Chan et al. 2001). In the animal resulting from the transduced oocyte, EGFP could be detected in several tissues by optical techniques under UV light and also by means of PCR. Whether the germ cells were also transgenic, was not reported. In the study by (Wolfgang et al. 2001) the expression of the EGFP transgene was restricted to extra-embryonic tissues like the placenta, the amnion and the umbilical cord. Noteworthy, this study also demonstrated the presence of antibodies directed against the EGFP protein in the pregnant female harboring the transgenic fetus. However, the transgenic fetus was not rejected. This finding was of importance for the development of the whole field of NHP transgenesis.

In 2008, Yang and colleagues reported an essential step towards the goal of genetically modified NHP disease models (Yang et al. 2008). They showed for the first time that pathogenic DNA sequences can function in genetically modified NHP. They introduced a mutated form of exon 1 of the human *Huntingtin* (*HTT*) gene into rhesus macaques. Huntington's disease (HD) is a so-called trinucleotide repeat disorder (Orr and Zoghbi 2007). Importantly, the disease-causing mutation is well-defined: Individuals with more than 40 CAG triplets (each triplet encoding glutamine) in the first exon of *HTT* develop clinical features of HD with 100% penetrance. The patients´ symptoms include severely impaired motoric coordination and a cognitive decline associated with psychological problems. Pathologically, atrophy of the cerebral cortex can be observed. Most patients die within 20 years after appearance of the first clini-

cal symptoms. HD typically becomes evident between 40-60 years of age, and there is no cure of the disease. Yang and colleagues introduced different versions of exon 1 of the *HTT* gene into rhesus monkey oocytes covering a trinucleotide repeat range from 29 to 88 repeats. Indeed, the monkeys with extended CAG repeats (>40) showed histopathological and clinical hallmark features of HD (Yang et al. 2008), impressively demonstrating the general feasibility of the generation of transgenic NHP disease models.

Another study confirmed the feasibility of rhesus monkey transgenesis. Niu and colleagues (2010) transduced EGFP via a simian immunodeficiency virus-based vector into early cleavage stage embryos, which were obtained by *in vitro* fertilization. Importantly, a careful comparative follow-up of the transgenic animals with control animals revealed no differences between both groups. Also the postnatal and prepubertal development of stature and body weight was normal in the transgenic monkeys suggesting no adverse effects of the transgene itself on the postnatal development.

The first transgenic non-macaque NHP species was the common marmoset monkey (*Callithrix jacchus*). Sasaki and colleagues (Sasaki et al. 2009) showed successful EGFP transgenesis of this species. They injected EGFP expressing lentiviral vectors into the subzonal space of preimplantation embryos at the blastocyst stage. After delivery of vital offspring, the transgene was found to be expressed in a number of somatic and extramebryonic tissues. However, the major novelty of this study was the first demonstration of germline transmission of the transgene.

Taken together, these studies showed that EGFP transgenesis is compatible with (i) implantation of the embryos (i.e. there is no rejection of the implanted transgenic embryo by the surrogate mother´s immune system), (ii) normal pre- and postnatal somatic development as well as with (iii) production of fertilization-competent transgenic germ cells. Furthermore and probably most important, hallmark characteristics of HD, an incurable and lethal human disease, can be induced in rhesus monkeys by lentivirus-mediated expression of a pathogenic fragment of the human *HTT* gene. This pathological modification is also germline transmissible (Putkhao et al. 2013).

5.2 *In vivo* gene targeting to generate loss of function alleles

Recently, two breakthrough papers were published reporting the first targeted loss of function mutations in NHP (Liu et al. 2014, Niu et al. 2014). Targeting

was achieved by the delivery of TALENs to rhesus and cynomolgus monkeys (Liu et al. 2014) and by the CRISPR-Cas9 components to cynomolgus monkeys (Niu et al. 2014).

Liu and colleagues targeted the methyl CpG binding protein 2 gene (*MECP2*) which is an X chromosome linked gene frequently mutated in Rett syndrome (Amir et al. 1999). The Rett syndrome is a severe progressive neurological disorder resulting in mental retardation. Although the Rett syndrome is a rather rare disorder compared to Parkinson's and Alzheimer's disease, its clinical relevance is highly significant due to the severity of the symptoms. Since access to relevant human tissues for the investigation of the Rett syndrome is extremely limited, the genetically modified macaque is most likely a very useful model for the investigation of cell and histopathological aspects of the Rett syndrome and possibly also a very good model for the investigation of therapeutic strategies.

The simultaneous functional deletion of two genes in cynomolgus monkeys was achieved by Niu et al. (2014) using the CRISPR-Cas9 system. The targets were the Peroxisome proliferator-activated receptor gamma (*PPARG*) gene encoding the PPAR-γ protein and the recombination activating gene 1 (*RAG1*) gene encoding the RAG-1 protein. PPAR-γ plays an important role in the regulation of many metabolic processes and has been implicated in the development of several disorders including such highly relevant ones as obesity and diabetes (Lazar 2005). RAG-1 is a protein involved in antigen receptor gene cleavage and rejoining for the generation of antibody diversity, which is an essential process for normal immune function (Nishana and Raghavan 2012). Beside the technical proof of principal, this study also provides the founder animals for the study of two biomedically highly relevant genes in a NHP context.

Importantly, off-target effects, i.e. the modification of unintended genomic sites, have not been detected in this study (Niu et al. 2014). This is of major relevance since unintended, adverse mutations could interfere with the aim of a study. Further improvements enhancing the genome editing specificity and versatility of the CRISPR/Cas9 system are already under development (e.g. Hsu et al. 2014, Ran et al. 2013).

Some of the most relevant disorders such as obesity / type 2 diabetes (Joost 2010) and heart disease (D'Alessandro et al. 2012) are multifactorial, i.e. the disorder cannot be attributed to a single gene. Instead, multiple genes and probably also additional cues like environmental factors cause and modulate the disease. Therefore the simultaneous modification of two or more genes, as it

Figure 1:
The common marmoset monkey (*Callithrix jacchus*, left) and the rhesus monkey (*Macaca mulatta*, right).

was recently also shown in the mouse (Wang et al. 2013), is of tremendous importance for the future study of complex genetic disorders in genetically modified animal models, including NHP. Furthermore, the versatility of the CRISPR/Cas9 system allows, as shown in mice (Yang et al. 2013), also the site-specific insertion of DNA fragments up to several kb in size and the insertion of predefined point mutations (Wang et al. 2013), offering additional options of modelling diseased human states.

The smartest aspects of the CRISPR-Cas9 technology include the relative technical ease and the rapidness of the procedure in comparison with classical mouse genome editing procedures and also the TALEN technology. This makes the pioneering CRISPR-Cas9 technology (and related future technologies) extremely promising with regard to the establishment of cohorts of genetically modified NHP which will boost the preclinical development of therapeutic strategies against some of the most severe scourges for mankind.

Table 2:
Biological key characteristics of macaques and the marmoset monkey relevant for genetic modification. Some data listed in this table was taken from: pin.primate.wisc.edu/factsheets

	Macaque		Marmoset
	Rhesus	Cynomolgus	
Body weight (kg)	♂ 7.7 (average) ♀ 5.3	♂ 4.7-8.3 (range) ♀ 2.5-5.7	0.3-0.5
Gestation (days)	164	165	148
Number of litters/year	1	1	2
Seasonality of reproduction in captivity	yes	no	no
Number of offspring/litter	1	1	2 (to 3 in captivity)
Age of sexual maturity (years)	♂ 4 ♀ 3	♂ 7 ♀ 4	~1,5

6. The choice of the NHP species

So far, two macaque species and the common marmoset monkey (*Callithrix jacchus*) have been genetically modified. The macaque species [rhesus macaque (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*)] belong to the old world monkeys. They are phylogenetically closely related and therefore share many biological characteristics. However, while rhesus macaques are seasonal breeders, cynomolgus monkeys show a constant reproductive activity during the cycle of the seasons (Table 2). Rhesus macaques further have a significantly higher average body weight than cynomolgus monkeys. In contrast to these very similar "sibling macaque species", the marmoset monkey exhibits many highly different biological features (Table 2). It is a new-world monkey with a much bigger phylogenetic distance to the human than the macaque (Finstermeier et al. 2013). This is also reflected by generally higher DNA sequence similarity between human and macaque than between human and marmoset (Marmoset Genome Sequencing and Analysis Consortium 2014, Peng et al. 2014). However, the marmoset has several highly significant practical and biological advantages: (i) housing costs are significantly lower for marmoset monkeys than for macaques. (ii) Marmoset monkeys do not have any zoonoses

while macaques can transmit the highly lethal Herpes B virus to humans. (iii) Very importantly, the generation time of marmosets is significantly shorter than that of macaques. (iv) Marmosets naturally give birth to twins, in captivity they often also deliver triplets. Macaques, in contrast, deliver only singletons, and multiples are at risk of severe birth complications. This is important with regard to the number of embryos that can be transferred to surrogate mothers with a good chance of delivering healthy offspring from the transferred embryos. (v) Finally, marmosets (at least in captivity) do not show seasonal reproduction as rhesus macaques do. Therefore, reproductive biology studies with marmosets can be done independent of the season. Altogether, marmosets have a significantly higher fecundity, which is of utmost importance for projects aiming at germline-transmissible genetic modifications. In conclusion, the specific reproductive and general biological characteristics of the different NHP species should be considered very carefully particularly in the light of the long-term character of studies involving genetic modification of NHP.

7. Conclusion and outlook

Accurately predefined gene loss of function, gene gain of function and gene function modulating studies are becoming possible in NHP. This promotes the idea of carefully designed NHP models to study and tackle human diseases (Cyranoski 2014, Pennisi 2014, Shen 2013), including complex polygenic disorders. This could be of relevance with regard to numerous medically and socio-economically highly relevant human genetic disorders. Genetically modified NHP models will provide invaluable and meaningful novel insights into pathomechanisms and thereby will promote and inspire preclinical research on currently incurable human diseases. "Research involving nonhuman primates has played a vital role in many of the medical and scientific advances of the past century" (Phillips et al. 2014). In continuation of this, the invention of precise and accurate genome modification in NHP will most likely play a vital role in many of the medical and scientific advances of the *present* century. The potential of genetically modified NHP models for biomedicine is considered immense.

8. Note

This review focuses on the biological, medical and technological aspects of genetic modifications of NHP. The author is aware of the fact that projects aiming at the genetic modification of NHP also have profound ethical implications –

for the patients potentially benefitting from genetically modified NHP as well as for the modified NHP itself. However, since the ethical aspects were not the topic of this paper, they were not discussed here.

9. Acknowledgements

10. References

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20th Primate Symposium – Concluding Remarks

Dr. Andrea Greiter-Wilke

The 20th Primate Symposium took place at the beautiful Hotel-Residence Klosterpforte in Marienfeld near Muenster, Germany. Friedhelm Vogel welcomed the numerous guests and introduced the four topics of the meeting: 1) Immunogenicity assessment – Challenges and opportunities, chaired by Shawn Heidel and Alison Wolfreys, 2) Neurotoxicity and CNS function, chaired by Mary-Jeanne Kallman and Oliver Czupalla, 3) Update on juvenile toxicity, chaired by Paul Baldrick and Randy Soltys, and 4) NHP disease and efficacy models, chaired by Lynn Anderson and Paul Germann.

The symposium was opened with the first session on *immunogenicity assessment*: Peter Lloyd's presentation on regulatory and scientific considerations highlighted the different types of anti-drug antibodies (ADAs) that could either prolong exposure or lead to loss of exposure, depending on the type of ADAs produced by the host. Measuring the ADA response was important for study interpretation, but was not predictive for humans ADA effects. He described the various assays available to assess immunogenicity and stressed the possibility to premedicate animals with steroids and/or antihistamines to avoid immune related adverse events. He concluded his presentation with a case study.

Similar points with regards to ADA production, PK assessment and study interpretation were also raised by Hishani Kirby. She highlighted the fact that in early development the required reagents for immunogenicity assays were not always available based on suitability. Wendy Halpern described a case study on the non-clinical safety evaluation of an anti-PDL1 antibody. The predominant side effects of drug treatment in cynomolgus monkeys were a 90% immunogenicity rate coupled with periarteritis and sciatic neuropathy. The presence of immunogenic effects at all test doses may be acceptable to the FDA if the magnitude of immunogenicity influencing the toxicity can be adequately described.

After the traditional Primate Symposium group photograph and coffee break, the second session focused on *the Evaluation of Neurotoxicity and CNS functions*. Jo Arezzo's presentation on neuropathies was webcast electronically. Preceded by a "technical bit of fun", it focused on different types of neuropathies,

their origin and potential diagnostic procedures. Dr. Arezzo reminded the audience not to forget the small fiber neuropathies and stressed the difference in anatomy between the dog and the monkey with regards to neurologic assessments. Neuroimaging was presented as a promising new technology for drug development.

Alessandra Giarola provided insights into the challenges of including safety pharmacology endpoints to evaluate CNS effects in repeat dose toxicology studies. Assessing CNS effects on Day 1 is usually not possible, since frequent manipulations of the animals for toxicokinetic blood sampling will prevent a meaningful behavioral evaluation. Various study designs, with staggered dosing to allow sufficient time for staff to perform these investigations several times a day, were discussed.

Michael Nader highlighted the value of the monkey as a model for non-clinical drug abuse liability assessment. Vascular access ports can be maintained in this species for several years, thereby enabling long-term use of trained animals for self-administration and drug discrimination tests. However effects in the monkey are not always similar to the ones seen in rats, certain drugs provoke different responses in both species.

The first day was concluded by a reception and barbecue dinner in the beautiful gardens of the Hotel Klosterpforte, allowing colleagues and friends to meet in a very relaxed atmosphere.

The first session of the second day provided an "*Update on juvenile toxicity*" in the NHP as an alternative species. Gerhard Weinbauer stated that most juvenile studies in NHPs were performed in the cynomolgus monkey and also, though rarely, in the marmoset. He laid out a comparison of the "organ age" between the cynomolgus and human and the typical design and animal age for a juvenile NHP study. He emphasized the value of different imaging techniques such as MRI and CT to assess organ development and toxicity and also discussed some case studies. Pauline Martin continued to describe the development of various organ systems in the cynomolgus monkey and focused on this species as a model in which to evaluate the toxicity of monoclonal antibodies. In most cases these are not cross-reactive in rodents or rabbits. She also provided an insight into the typical development of monoclonal antibodies and the fact that some even cross the placenta in NHPs. One might argue however that juvenile studies are not always necessary at all for developing monoclonal antibody therapies. A case study on physial dysplasia concluded her talk.

Wendy Halpern discussed considerations in the design of molecule-specific pre-clinical testing strategies in NHPs to enable pediatric clinical trials. Since tumors in juveniles are rarely similar to tumors in adults, knowledge of the mode of action of the drug in development is of high importance, especially in children between 2 and 12 years of age. Puberty in children may be variable in onset and manifestation; therefore especially the cardiovascular system among other organ systems may be sensitive for acute and delayed effects. Similarly, ADME properties can vary between adults and juveniles. She highlighted that for all these reasons histopathology needs be assessed in enhanced pre- and postnatal development (ePPND) studies to support pediatric development.

The last session of the symposium focused on *NHP disease and efficacy models*. Michael Nader described the CANTAB model for cognitive assessment and its application to Alzheimer's disease. CANTAB stands for "Cambridge Neuropsychological Test Automated Batteries" and was designed to adapt paradigms developed in animal models for use in humans; using computer-based nonverbal testing developed in rhesus monkeys. The advantage of the NHP in performing such tests is the high similarity in frontal cortex anatomy to humans compared to rodents, allowing various different tests to be performed in one animal. Several tests have been successfully employed in the rhesus monkey, such as working memory, discrimination and also cognition. Michael Nader reviewed the evaluation of cognition in naïve and cocaine-treated rhesus monkeys for the treatment of Parkinson's disease, where PET-imaging was of importance to assess the effects of cocaine on glucose uptake.

Ruediger Behr presented the potential of genetically modified NHP models for biomedicine. He described the differences between mice and monkeys with regards to the ease of genetic manipulation and, for Parkinson's research, identified that the genetically modified monkey is preferred over a purely pharmacological model. Genetic modification previously employed lentiviral transgenesis and embryo transfer; however the new CRISPR/cas9 genome editing technology allows predefined mutations and knockouts to be produced in one step. This substantially reduces the time and effort needed to obtain genetically modified animals.

As the final speaker, Richard Schroeder highlighted the NHP as a model for diabetes and the detection of cardiovascular abnormalities associated with this condition. He described different methods and biomarkers to assess vascular function and compliance in humans and how to transfer this methodology to NHPs. Rosiglitazone was discussed as an example being tested in the NHP.

The symposium was concluded by my closing remarks, thanking the organizers for a very successful meeting not only with regards to the excellent scientific contributions, but also memorable for its beautiful location as well as the social and the culinary highlights!

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TWENTIETH PRIMATE SYMPOSIUM

Primate Biologics Research
at a Crossroads

– PROGRAMME –

14:00   **Opening Remarks**
Friedhelm Vogel, Vice President, NHP Science and Supply
Strategy, Covance

SESSION 1
IMMUNOGENICITY ASSESSMENT: CHALLENGES
AND OPPORTUNITIES

14:10   **Introduction – Session Co-Chairs**
Shawn Heidel, Executive Director, Lead Optimization
Pharmacology and Toxicology, Covance
Alison Wolfreys, Director, Nonclinical Safety Evaluation,
UCB, Celltech
*Immunogenicity assessment is an inherent component of
biopharmaceutical drug development and ADA assessment is
critical for study interpretation. The purpose of this session is
to discuss how to assess and manage immunogenicity for study
relevance and specifically the role of NHPs in this context.*

14:15   **Immunogenicity: Regulatory and Scientific
Considerations**
Pete Lloyd, Head of DMPK-Biologics, Novartis

14:45   **Lost in Translation: impact of anti drug antibodies from
monkey to man (mAb therapeutics)**
Hishani Kirby, Director, Bioanalytical Sciences, UCB
Celltech

15:15   **Nonclinical Safety Evaluation of Anti-PDL1
(MDPL3280A) in the Context of High Immunogenicity**
Wendy Halpern, Senior Scientist/Pathologist, Genentech, A
Member of the Roche Group

**SESSION 2
EVALUATION OF NEUROTOXICITY AND CNS
FUNCTIONS**

16:15   **Introduction – Session Co-Chairs**
Mary-Jeanne Kallman, Director, Global Nonclinical
Neuroscience, Covance
Oliver Czupalla, Experimental Toxicology, Laboratory
Diagnostics, Bayer Pharma AG
*Neurological, behavioural, cognitive and CNS function assessments
are important during various preclinical development phases. If
NHP models are to be used, special expertise and appropriate
testing strategies are pivotal and will be addressed in this session.*

16:20   **Assessment of the Onset, Progress and Possible Recovery
of Compound-Induced Neuropathies in Nonhuman
Primates**
Joseph C. Arezzo, Professor of Neuroscience and Neurology,
Albert Einstein College of Medicine

16:50   **The Use of Primate CNS Evaluation for the
Characterization or Resolution of the Effects of Biologics**
Alessandra Giarola, Manager, CNS Safety Pharmacology,
GlaxoSmithKline

17:20   **Monkey Models of Abuse Liability**
Michael A. Nader, Professor, Wake Forest School of
Medicine

SESSION 3
JUVENILE TOXICITY ASSESSMENT UPDATE

9:00   **Introduction – Session Co-Chairs**
Paul Baldrick, Senior Director, Regulatory Strategy, Covance
Randy Soltys, Vice President, Drug Safety &
Pharmacometrics, Regeneron
*Addressing concerns for potential drug use in a pediatric
population is now an integral part of preclinical safety assessment.
This session will highlight options and challenges when NHPs are
used in juvenile toxicity evaluations, and also the relevance of the
NHP model.*

9:05   **Juvenile Toxicity Testing: Current Experience Using
NHPs**
Gerhard Weinbauer, Vice President, Global DART, Covance

9:35   **Considerations for the Development of Monoclonal
Antibodies for Pediatric Indications: Using a Weight of
Evidence Approach as an Alternative to Juvenile Toxicity
Testing in NHPs, Biologics Toxicology**
Pauline Martin, Senior Director, Biologics Toxicology,
Janssen R&D

10:05   **Considerations in the Design of Molecule-Specific
Preclinical Testing Strategies in NHP to Enable Pediatric
Clinical Trials**
Wendy Halpern, Senior Scientist/Pathologist, Genentech, A
Member of the Roche Group

SESSION 4
NHP DISEASE AND EFFICACY MODELS

11:05   **Introduction - Session Co-Chairs**
Lynn Anderson, Vice President, Global Animal Welfare and
Laboratory Animal Medicine, Covance
Paul Germann, Head Preclinical Safety Germany, AbbVie
Deutschland GmbH & Co KG
*Unlike for rodents, the choice of NHP models that are well
established for particular diseases is rather limited for efficacy
models. In this session, NHP model selections will be presented and
discussed in terms of relevance and translatability.*

11:10   **The CANTAB Models for Cognitive Assessment and
Application to Alzheimer's Disease**
Michael A. Nader, Professor, Wake Forest School of
Medicine

11:40   **Potential of Genetically Modified NHP Models for
Biomedicine**
Rüdiger Behr, Stem Cell Biology Unit, German Primate
Centre, Leibniz- Institute for Primate Research

12:10   **Nonhuman Primate Models of Obesity and Abnormal
Glucose Metabolism: Models for Identifying Disease-
Associated Cardiovascular Abnormalities**
Richard L. Schroeder, Lead Scientist and Study Director,
Lead Optimization Pharmacology, Cardiovasular and
Metabolic Disease, Covance

12:40   **Closing Remarks**
Andrea Greiter-Wilke, Head Safety Pharmacology,
F. Hoffmann-La Roche AG